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EFFECTS OF X-IRRADIATION ON AMOUNT AND COMPOSITION OF NUCLEIC ACIDS IN LIVER [1]

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TWO FIGURES

INTRODUCTION

One of the most striking of the biological effects of ionizing radiations is that produced on the hereditary materials of the cell. In view of present concepts regarding the role of nucleic acids in genetic mechanisms, it is important to understand the effects of ionizing radiations on nucleic acids. The exact nature of these effects, however, is not well established. Current literature presents conflicting reports regarding the changes induced in the nucleic acids of various tissues by X-irradiation. Several workers, approaching this problem by the use of chemical and cytochemical methods, have reported decreases either in the total content or in the rate of synthesis of pentose nucleic acid (PNA) or desoxypentose nucleic acid (DNA) (Ely and Ross, '48; Abrams, '51; Ahlstrom et al., '44). Petrakis et al. ('49), however, working with rat liver epithelium, observed an initial rise in the quantity of both nucleic acids shortly after irradiation, followed by a drop to below the control level.

H. Harrington and Lavick ('51a, b), in a study of effects of irradiation on the thymus gland, found no change in the

[1] This work was supported in part by a research grant (RG-149) from the National Institutes of Health, U. S. Public Health Service, to Dr. Berwind P. Kaufmann.

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content of DNA phosphorus per nucleus, although there was a decrease in the total DNA phosphorus of the gland. In a study of embryonic grasshopper nuclei subjected to high doses of x rays, N. J. Harrington and Koza ('50) confirmed the finding that there is no decrease in DNA content per nucleus, as measured by the Feulgen stain, although on the basis of staining reactions with methyl green they suggested that irradiation produced a depolymerization of the DNA. Moses et al. report, however, that if high intensity irradiation of *Trillium* produces any change in DNA, it is an increase rather than a decrease in the degree of polymerization. Kaufmann et al. ('51), in a similar study made on onion root tips, reached the same conclusion.

Mitchell ('42, II, III) suggested that the accumulation of PNA in the cytoplasm and the loss of DNA from the nucleus, which he observed in tissues of humans exposed to therapeutic doses of x and γ rays, is due to inhibition of the reduction of ribo- to desoxyribonucleotides in the nucleus. De Niccola ('50a, b), on the other hand, concluded that irradiation results in the inhibition of DNA synthesis and of a postulated conversion of DNA to PNA.

The present report describes a study, using chemical methods, of the effects of whole-body X-irradiation on the nucleic acids of mouse liver. Observations were made on the nucleic acids at various times after exposure to a constant dose of x rays. After determining that quantitative changes were produced, the question arose whether such irradiation produces, in addition to these quantitative changes, alterations in the type or composition of the nucleic acids as well. A search of the literature revealed no information on this point. Consequently, both pentose nucleic acid and desoxypentose nucleic acid were isolated from the livers of irradiated and control mice and subjected to a purine and pyrimidine analysis, using a method of quantitative paper chromatography. It was found that irradiation produced no change in the ratios of the various bases.

METHODS

Young adult male mice (32–42 days old) from a highly inbred C58 strain were used throughout.[3] For the studies on changes in DNA and PNA levels 60 mice were starved for 24 hours, irradiated with 600 r of hard x rays, and then permitted to feed *ad libitum* until sacrificed. Twelve control mice were starved 24 hours, allowed to feed for 24 hours, and then sacrificed. The mice were irradiated in groups of 6 in a rotating (7.5 r.p.m.) lucite cage with a lucite cover 1.74 mm thick. The cage was placed 44.5 cm from the tube, which was operated at 140 K.V. and 7 ma in a North American Phillips Norelco Searchray machine. The x rays were filtered through 0.13 mm of copper and 0.85 mm of aluminum. Treatment was given at a rate of 12.4 r per minute for a period of 48 minutes. Doses were measured with a Victoreen r-meter.

The irradiated mice were sacrificed in groups of 12 at 6, 12, 18, 24, and 48 hours after the midpoint of the irradiation period. They were killed by stunning, and were then decapitated and allowed to bleed for 5 minutes. The excised livers were minced with scissors, homogenized in 7.0 ml of distilled water at 0°C. in a PotterElvehjem apparatus equipped with a lucite pestle mounted on a stainless steel rod, and strained through 4 layers of gauze. Duplicate 1 ml aliquots of the strained homogenate were taken for dry weight determinations.

The method used for extracting the nucleic acids was a slight modification of Schneider's procedure ('45). To duplicate 2 ml samples of strained homogenate and of a one-half dilution of it, 5 ml of 0.6 N trichloracetic acid (TCA) at 0°C. was added. The resulting precipitate was centrifuged down at 1200 g for 10 minutes in a refrigerated centrifuge. The supernatant from this will be referred to as the acid-soluble fraction. The precipitate was resuspended in 5 ml of 0.6 N TCA and recentrifuged; this step was repeated once more. These washings were discarded. The washed precipitate was

[3] We are grateful to Dr. E. C. MacDowell who generously supplied these mice. They represented the product of over 90 generations of brother-sister matings.

then suspended in 10 ml of 0.3 N TCA and placed in a boiling water bath for 15 minutes, cooled to room temperature, and filtered through Schleicher and Schuell no. 575 paper.[4] The precipitate was discarded. The filtrate contains the nucleic acids and will be hereafter referred to as the hot TCA extract.

The DNA content of the hot TCA extract was determined according to the method of Dische ('30), using 1 and 2 ml aliquots (1 ml aliquots were adjusted to 2 ml with boiled TCA) and 4 ml of the diphenylamine reagent. Colors were read at 600 mμ. Standards were prepared by extracting with hot TCA a commercial sample of DNA (Dougherty Chemical Co.), which contained 7.11% phosphorus and 69% DNA according to assay against a pure sample of desoxyribose.[5] The DNA values obtained were calculated in terms of the phosphorus content of the standard, and expressed as milligrams of DNA phosphorus per gram dry weight of homogenate.

The PNA content of the hot TCA extract was determined as pentose by the method of McRary and Slattery[6] ('45), using 0.5 and 1 ml aliquots (adjusted to 2 ml with boiled TCA) and 6 ml of orcinol reagent. Depending on the intensity, colors were read at 600 or 670 mμ. Xylose was used as the standard, and the results were expressed as milligrams PNA phosphorus per gram dry weight of homogenate. The necessary conversion factor was obtained from a commercial yeast nucleic acid sample, purified according to the method of Woodward ('44); it contained 62.3 μg phosphorus per micromole xylose equivalent.[7]

Corrections were made for interference by DNA in the determination of PNA by the orcinol method. Experimentally,

[4] Various filter papers, when extracted with hot TCA, yielded material that reacted as PNA in the orcinol reaction. This interference may be kept to a minimum by using S. and S. no. 575 and cooling the TCA extract before filtration.

[5] The value for the DNA content was supplied through the courtesy of Dr. Seymour S. Cohen.

[6] For convenience, one part of 4% orcinol in water and 19 parts of 0.075% $FeCl_3 \cdot 6\,H_2O$ in 30% HCl were mixed fresh daily.

[7] This sample was kindly supplied by Dr. M. R. McDonald.

1 mg of DNA phosphorus gave as much color as 0.189 mg PNA phosphorus.

For each mouse, two levels of homogenate and two dilutions of each extract were employed. In the case of DNA, an average of the three highest values was used (the amount of color produced from a 1 ml aliquot of the hot TCA extract derived from 1 ml of homogenate was considered too small to be an accurate measure). For PNA an average of all 4 determinations was used. In all cases good agreement was obtained between analyses at the different dilutions of homogenate and extract. All samples from one time group were processed together, along with a set of standards. All colors were determined with a Coleman Jr. spectrophotometer.

Ultraviolet absorption data were obtained as follows. One milliliter of the hot TCA extract and 1 ml of the acid-soluble fraction, both derived from the 2 ml aliquot of homogenate, were each diluted with 10 ml of water and read against appropriate blanks in a Beckman spectrophotometer at 264 mμ. In addition, for two hot TCA extracts and two acid-soluble fractions, picked at random from each group of mice, complete absorption spectra were obtained over the range 240–300 mμ at intervals of 5 mμ. The absorption spectra, when calculated as the fraction of the extinction at 260 mμ, agreed within 2% for each pair of extracts.

In the studies of DNA and PNA composition the techniques of irradiation and preparation of the homogenates, as well as the colorimetric analyses for PNA and DNA, were identical with those already described.

Two groups of 12 mice each were sacrificed at 18 and 48 hours, respectively, after exposure to 600 r of hard x rays. Twelve non-irradiated mice were used as controls. The excised livers were pooled by fours within each group, and each set of pooled livers was homogenized separately in 40 ml of distilled water at 0°C.

Fractionation of the homogenates was carried out using the Schneider ('46) modification of the procedure of Schmidt and

Thannhauser ('45). To 30 ml of homogenate was added 6 ml of 3.0 N trichloracetic acid at 0°C., and the resulting precipitate was centrifuged down and washed twice with 30 ml of 0.6 N TCA at 0°C. Phospholipids were removd from the precipitate by extraction first with 35 ml of cold 95% ethanol, followed by two 15-minute extractions with 30 ml each of 95% ethanol at 70°–75°C. The remaining sediment was dissolved in 30 ml of 1.00 N KOH and incubated 18 hours at 36°C. It was then made up to 50 ml with 1.00 N KOH (about 45 ml total of KOH solution was used for each sample); and for each volume of KOH used, 0.20 volumes of 6.00 N HCl and 0.133 volumes of 3.00 N TCA were added. The resulting precipitate was centrifuged down, and the supernatant saved as the PNA-containing fraction. The remaining precipitate was washed once with 30 ml of an HCl-TCA mixture (100 ml H_2O plus 20 ml 6 N HCl plus 13.3 ml 3 N TCA), and once with 30 ml of 0.6 N TCA. The DNA-containing fraction was obtained by extraction of the residue with 30 ml of 0.3 N TCA in a boiling water bath for 20 minutes followed by filtration through Schleicher and Schuell no. 575 filter paper.

Desalting of the PNA fraction was carried out by precipitating the potassium as the perchlorate and removing the chloride ion via HCl. Forty milliliter samples were boiled down until the KCl just precipitated out, and this procedure was repeated after the addition of 20 ml of water. A few drops of water were then added to bring the KCl back into solution, and 5.40 ml of a 5.90 N solution of perchloric acid and 10 ml of 95% ethanol were added. This represented 1.03 equivalents of perchlorate ion for each equivalent of potassium. Flasks were stored at —25°C. overnight and the precipitated salt was removed by filtration. The filtrates were boiled down to 4–5 ml, again chilled overnight, and filtered directly into 10 × 75 mm soft glass test tubes. These filtrates were taken to dryness, using a boiling water bath and a stream of clean air. Recovery experiments showed that no PNA was lost by this procedure.

The DNA fraction was prepared by boiling 20 ml down to 3–4 ml, adding 20 ml of water, and again evaporating the solution down to 2–3 ml. It was then transferred to a 10 × 75 mm soft glass test tube and taken to dryness as above.

Both DNA and PNA samples were hydrolyzed by adding 0.3 ml of 70% perchloric acid (11.8 N), sealing the tubes, and immersing them in a boiling water bath for one hour. For convenience, 0.15 ml of water was added after hydrolysis; in some cases, to make pipetting easier, the PNA hydrolysates were filtered through Whatman no. 1 paper before use.

Descending chromatograms were run at 18°C. according to the method of Wyatt ('51). The solvent contained 59% (v/v) c.p. isopropanol and was 2.3 N in HCl. Whatman no. 1 paper was used, and the sheets were washed with water before use in order to reduce blank values. Six lanes, 3 cm wide, were used on each chromatogram; 4 contained 0.010 ml aliquots of hydrolysate, and two were used as blanks. A total of 8 spots on two chromatograms was run for each sample. Spots were identified by using an ultraviolet lamp (Chargaff et al., '51). The spots were marked, cut out, and eluted overnight at 38°C. with 4.0 ml of 0.1 N HCl. Eluates were read in a Beckman spectrophotometer (Wyatt, '51).

RESULTS

Quantitative changes. The results obtained when the nucleic acid content of mouse liver is studied as a function of time after irradiation with a 600 r dose of hard x rays are shown in figure 1. The fact that the ultraviolet absorption of the hot TCA extract (at 264 mμ), which depends upon changes in the quantities of the nitrogenous bases, was in agreement with the colorimetric determinations of DNA and PNA, based upon the sugar components, provides an independent check of the validity of the observations. The extent of this agreement is shown in table 1, where the observed extinction is compared with that calculated from the nucleic acid content as determined by sugar analyses.

The most striking thing about these results is the parallel behavior of DNA and PNA. This is strongly suggestive of common or similar steps in the metabolism of both nucleic acids; that is, reactions whose catalysts are affected in an identical or almost identical fashion by x rays, particularly with respect to the time after irradiation at which the effects occur. The similar results obtained with the two nucleic ac-

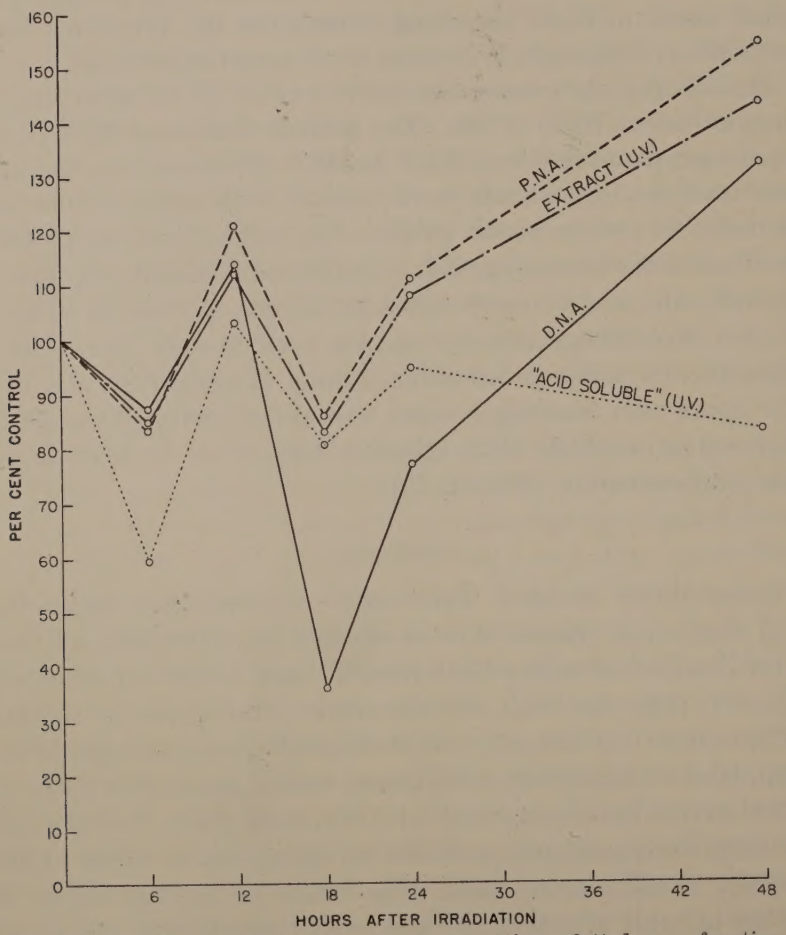

Fig. 1 The nucleic acid constituents of mouse liver plotted as a function of time after irradiation. The data are expressed as percentages of the control values.

ids is strong evidence against the hypothesis advanced by Mitchell ('46, II, III), and by de Niccola ('50a, b), that irradiation results in a transformation of nucleic acid from one type to another.

With regard to the question of whether irradiation effects an increase or decrease in the nucleic acids of a cell, these

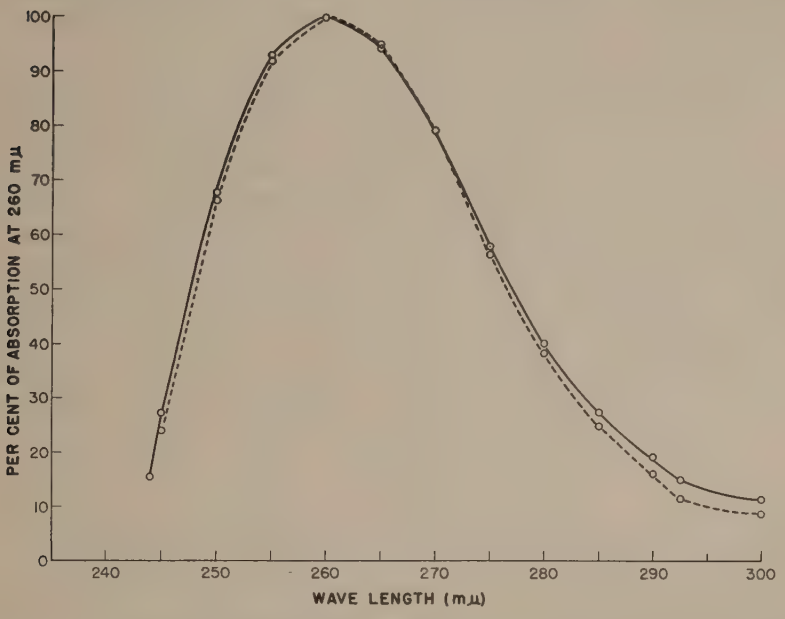

Fig. 2 Absorption spectra of the "acid-soluble" fraction obtained from control and 18-hour mice. Data are expressed as percentages of the absorption at 260 mμ to permit easier comparison. 0 —— 0 curve for control mice. 0 – – – 0 curve for 18-hour mice.

data show both a higher and a lower content, at different times after irradiation. It is to be expected that the exact time intervals at which increases or decreases occur would depend upon both the particular tissue studied and the dosage level employed.

Figure 1 also shows the light absorption at 264 mμ of the acid-soluble fraction. The components of this fraction that might be expected to contribute to the light absorption at

264 mμ are purines and pyrimidines, both conjugated and free, tyrosine, tryptophane, and low molecular weight peptides containing these two amino acids. The absorption spectra of the control and 18-hour samples of the acid-soluble fraction are given in figure 2. By the absence of any discernible deviation from a smooth curve in the region of 280 mμ it can be seen that amino acid containing materials do not contribute significantly to the light absorption of the acid-soluble fraction.

Considering, then, that the intensity of the 264 mμ absorption in the acid-soluble fraction represents the level of purine and pyrimidine bases, both conjugated and free, it is of great interest that the fluctuations of this curve in figure 1 follow those of the nucleic acids. If radiation sensitive reactions were involved in the interconversion of nucleic acids and nucleotides, it would be expected that the levels of nucleic acids and nucleotides would vary reciprocally, since substrates of a reaction tend to accumulate as their conversion to a product is inhibited. The fact that the levels of nucleic acids and of the free nucleotides vary similarly, may be interpreted in two ways. The radiation sensitive reaction may precede the formation of nucleotides or other ultraviolet absorbing materials; or the changes in liver may reflect the release or mobilization of nucleic acid constituents by other tissues of the body. In the latter case, the liver would be functioning as a nucleic acid "reservoir."

In the work discussed results have been expressed as percentages of the same quantity in the control. A compilation of the actual data is given in table 1. The values of dry weight per milliliter of homogenate indicate no marked loss or uptake of water by liver after irradiation, except possibly in animals from the 48 hour group.

Changes in composition. Table 2 presents the results of DNA and PNA analyses of the isolated fractions to show the extent to which cross contamination occurs. It is apparent that contamination of the PNA fraction by DNA is not serious, although contamination of DNA by PNA is significant,

TABLE 1

HOURS AFTER IRRADIATION	NO. OF MICE	DNA PHOSPHORUS		PNA PHOSPHORUS		ULTRAVIOLET ABSORPTION OF HOT TCA EXTRACTS (observed)	ULTRAVIOLET ABSORPTION OF HOT TCA EXTRACTS (calculated)[3]	ULTRAVIOLET ABSORPTION OF ACID SOLUBLE FRACTION	AVERAGE DRY WEIGHTS OF 1 ML HOMOGENATE
		mg/gm dry weight[1]	P[2]	mg/gm dry weight[1]	P[2]	O.D. 264/gm dry weight/ml[1]	O.D. 264/gm dry weight/ml	O.D. 264/gm dry weight/ml[1]	mg[1]
0 (Control)	12	0.546 ± 0.014	.0310	2.28 ± 0.04	.0070	795 ± 20	876	221 ± 4.4	51.6 ± 0.97
6	10	0.474 ± 0.024	.0016	1.91 ± 0.10	.0004	681 ± 35	739	129 ± 4.9	49.7 ± 2.15
12	12	0.629 ± 0.026	< .0001	2.75 ± 0.13	.0002	890 ± 41	1048	228 ± 14.9	48.0 ± 1.39
18	11	0.200 ± 0.015	< .0001	1.96 ± 0.05	.0004	658 ± 29	671	178 ± 6.6	51.0 ± 2.02
24	12	0.421 ± 0.013	< .0001	2.58 ± 1.08	< .0001	863 ± 35	932	209 ± 6.1	46.0 ± 0.86
48	12	0.720 ± 0.018		3.51 ± 0.04		1136 ± 23	1313	183 ± 12.4	38.6 ± 0.79

[1] ± standard error of mean = $\sqrt{\Sigma d^2/N(N-1)}$.

[2] The probability that the difference between two adjacent values is due to chance.

[3] Calculated from DNA and PNA phosphorus data. Extinction coefficients at 264 mμ of 302/mg P/ml for DNA and 311/mg P/ml for PNA were used. These values were obtained by putting samples of DNA and PNA through the same analytical procedure used in the irradiation experiments.

amounting to 22% of the total in one case. In chromatograms in which there was a several fold excess of thymine over uracil, however, the uracil spot coincided with that for thymine, so that the values given for thymine are probably too high.

TABLE 2

DNA and PNA contents of nucleic acid fractions isolated from livers of control and irradiated mice

FRACTION	HOURS AFTER IRRADIATION		PNA PHOSPHORUS	DNA PHOSPHORUS
			(mg/gm dry wt.)	*(mg/gm dry wt.)*
PNA	Control	I	2.95	0.20
		II	3.02	0.23
		III	2.36	0.16
	18	I	2.19	0.19
		II	2.32	0.16
		III	2.10	0.18
	48	I	3.28	0.19
		II	3.46	0.20
		III	3.33	0.21
DNA	Control	I	0.041	0.410
		II	0.050	0.385
		III	0.034	0.305
	18	I	0.011	0.327
		II	0.022	0.290
		III	0.043	0.238
	48	I	0.058	0.351
		II	0.066	0.235
		III	0.076	0.277

Table 3 gives the results of the chromatograms for the various samples. No differences are detectable, and so it must be concluded that, within the limits of the experimental test, there is no change in the composition of the nucleic acids of mouse liver after whole-body exposure to 600 r of hard rays.

The authors wish to acknowledge their debt to Dr. B. P. Kaufmann and Dr. M. R. McDonald for their advice and as-

TABLE 3

Composition of nucleic acids isolated from livers of control and irradiated mice

HOURS AFTER IRRADIATION		DNA				PNA			
		Guanine	Adenine	Cytosine	Thymine	Guanine	Adenine	Cytosine	Uracil
Control	I	0.24	0.31	0.18	0.27	0.35	0.20	0.15	0.30
	II	0.20	0.32	0.22	0.26	0.34	0.22	0.16	0.28
	III	0.23	0.28	0.25	0.25	0.33	0.20	0.15	0.32
		(0.22)	**(0.30)**	**(0.22)**	**(0.26)**	**(0.34)**	**(0.21)**	**(0.16)**	**(0.30)**
18	I	0.23	0.31	0.18	0.28	0.35	0.19	0.17	0.29
	II	0.24	0.30	0.20	0.26	0.36	0.21	0.16	0.27
	III	0.25	0.29	0.23	0.22	0.33	0.19	0.19	0.29
		(0.24)	**(0.30)**	**(0.20)**	**(0.25)**	**(0.34)**	**(0.20)**	**(0.17)**	**(0.28)**
48	I	0.23	0.29	0.21	0.27	0.34	0.20	0.18	0.29
	II	0.27	0.30	0.21	0.22	0.32	0.20	0.21	0.28
	III	0.24	0.28	0.24	0.24	0.35	0.20	0.15	0.30
		(0.25)	**(0.29)**	**(0.22)**	**(0.25)**	**(0.33)**	**(0.20)**	**(0.18)**	**(0.29)**
Average		0.24	0.30	0.21	0.25	0.34	0.20	0.17	0.29

Results are expressed as mole fractions of each base. Figures in parentheses are the group averages.

sistance throughout the course of this investigation; and to Mrs. Graydon Vanderbilt and Miss Rebecca Wood for their technical assistance.

SUMMARY

1. The effect of whole body irradiation by hard x rays on the nucleic acids of mouse liver was observed at various times after irradiation with a fixed dose of 600 r.

2. The changes observed in the levels of nucleic acid were qualitatively identical for both DNA and PNA; that is, the levels of both rose and fell together.

3. There was an initial small drop, followed by a recovery at 12 hours to levels above those of the non-irradiated controls. A major drop occurred about 18 hours after exposure. This was followed by another rise, until at 48 hours after exposure the levels were well above those of the control values.

4. Evidence was obtained that the reactions affected by irradiation are not involved in the interconversion of nucleic acids and free nucleotides. The sensitive reaction may precede the formation of nucleotides or other ultraviolet absorbing material, or the changes observed in liver may result from its function as a nucleic acid "reservoir."

5. The purine and pyrimidine contents of DNA and PNA isolated before and after exposure were determined by quantitative paper chromatography. With a dosage of 600 r, animals sacrificed 18 and 48 hours after exposure showed no change in nucleic acid composition when compared with non-irradiated controls.

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THE EFFECT OF GLUCOSE AND pH UPON POTASSIUM LOSS FROM RABBIT RED CELLS

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FOUR FIGURES

Parpart and colleagues ('47) have reported observations on potassium maintenance in human red cells in buffered solutions when these cells are stored up to 4 weeks at different pH values with and without 0.5% glucose. Prior to this report no systematic studies of these two variables as affecting K loss from red cells have been found in the literature. Maizels ('35), Davson ('40), Solomon, Hald and Peters ('40), and Danowski ('41) have all considered one or more aspects of the problem of K loss from different species of mammalian red cells. Harris ('41) using human red cells observed greater retention of K by the cells in suspensions containing added glucose than in control suspensions. He also measured final pH values but made no study of the relations between glucose and pH as they affected K loss.

Other workers (see Ponder, '49) have measured K losses from mammalian red cells with only an incidental consideration of the constancy of the pH or of the glucose levels of the suspensions. A variety of reports in recent years on K-Na exchanges in red cells touch upon the present work indirectly and will be discussed in a subsequent paper. The fact that the end products of the active glucose metabolism of the red cells of many mammals is two molecules of lactic acid for

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each glucose molecule and the large changes in ion concentration that thus occur have been neglected in previous studies in this field.

The work to be reported here was designed to extend the observations of Parpart et al. ('47) using fresh rabbit red cells at temperatures of 35°C. The results indicate that pH has a pronounced effect on K retention, over-riding the effect of glucose at low pH levels, even though the rate of glucose utilization did not vary.

METHODS

Fresh rabbit blood, drawn by heart puncture, defibrinated and maintained under sterile conditions was used throughout. Two general types of experimental procedure were used.

Type 1 method: 20 ml of whole blood was centrifuged and 8 ml of the serum replaced with 8 ml of 0.11 M phosphate buffer at the desired pH. A modification of this procedure consisted of the replacement of the phosphate buffer at 6 hour intervals in an effort to maintain a constant pH. Such replacement took into account the amount of the whole suspension removed for analysis at each sampling time. The phosphate red cell suspensions were incubated at 35°C. for periods up to 50 hours. Glucose when added was added as a sterile solid.

Type 2 method: 10 ml of whole blood were added to 200 ml of a sodium chloride-phosphate buffered solution. This solution was prepared by mixing 350 ml of 1% NaCl with 150 ml of 0.11 M phosphate; the buffer contained only the sodium salts. In both types of procedure, samples of the red cell suspension were taken at intervals throughout the incubation period. The pH of the suspensions was measured by a glass electrode. The glucose concentrations of the suspensions were determined by a modification of the Hoffman ('37) method. In those experiments in which the red cell volume was small in comparison with the volume of the supernatant, only the glucose concentration of the supernatant was determined. The suspended cells were packed in the air turbine centrifuge (Parpart and Green, '51) and after removal of the super-

natant, carefully measured volumes of packed cells were hemolyzed in distilled water for K and Na analysis in the Perkin-Elmer flame-photometer. Cell volumes were determined from the spectrophotometric measurement of the hemoglobin concentrations in the laked cell solutions. All K and Na concentrations were corrected to the original cell volume (see Parpart and Green, '51). Inappreciable amounts of hemolysis were observed in the cell suspensions even after 48 hours of incubation.

RESULTS

When glucose is added to a red cell suspension in concentrations of 500 mg % and the K loss from these cells followed during a 36 hour incubation period at 35°C. the results shown in figure 1 are obtained. Type 1 method was used. In this

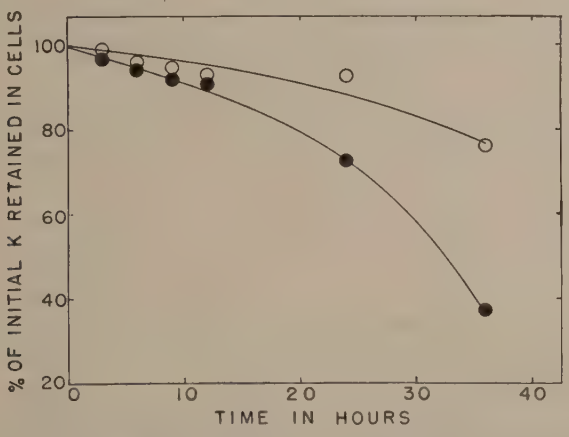

Fig. 1 Plot of potassium loss with time from rabbit red cells exposed to added glucose (open circles) and no glucose added (solid circles), under conditions of unequal pH change.

graph the cell K loss from the suspensions to which glucose was added is compared with that from cells in suspensions to which no glucose was added. It would appear that the addition of glucose to the medium is associated with K retention in these cells. However, some question about this interpretation is aroused by a consideration of table 1 which lists glu-

cose utilization and pH values of the cell suspension during the course of the experiment.

Table 1 shows a marked difference in the pH of the two cell suspensions existed during the greater part of the incubation period. As a consequence two uncontrolled variables were involved instead of one. It is obvious that the divergence in rate of K loss in the two curves (fig. 1) begins at about the time that an appreciable difference in the pH of the two suspensions develops (table 1).

When the experiment reported in figure 1 is repeated under conditions in which the pH of the two suspensions is maintained parallel and approximately the same by replacing the

TABLE 1

TIME IN HOURS	ADDED GLUCOSE SUSPENSIONS TOTAL GLUCOSE USED		NO GLUCOSE SUSPENSIONS TOTAL GLUCOSE USED	
	mg %	*pH*	*mg %*	*pH*
0	0	7.23	0	7.25
3	50	7.10	33	7.16
6	85	7.03	40	7.16
9	110	7.00	37	7.17
12	135	6.93	40	7.14
24	270	6.70	44	7.16
36	360	6.40	40	7.02

buffer in the two suspensions at intervals of 6 hours, the rate of K loss in the two suspensions is not strikingly different as shown by the curves in figure 2. Both figures 1 and 2 are plotted on the same scale and it is to be noted that for the first 24 hours the curves in figure 2 have essentially the same slope as their counterpart curves in figure 1. Examination of figure 2 during the 24 to 48 hour period shows that the rate of K loss from cells in the suspension with a low concentration of *"added-glucose"* decreases and becomes less than that in cells from the suspension with a high concentration of *"added-glucose."*

Table 2 provides additional information about this second experiment. The suspensions with high concentration of

"added-glucose" utilize about twice as much glucose as did those with low concentration during the same time. The pH values, shown by the table, while not the same are quite parallel, particularly after the first 12 hours of the experiment. The

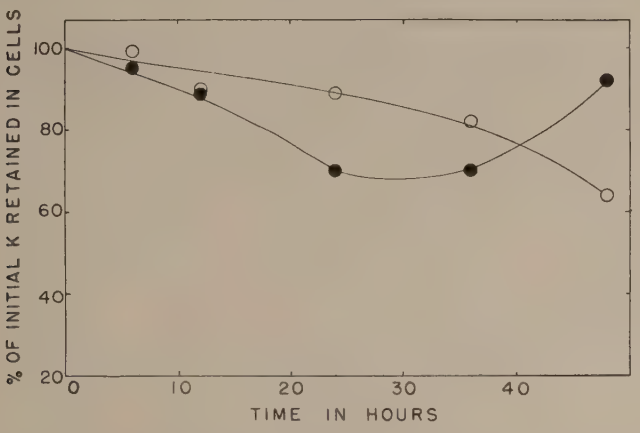

Fig. 2 Plot of potassium loss with time from rabbit red cells exposed to high (open circles) and low (solid circles) concentrations of glucose under conditions of approximately equal pH change.

TABLE 2

TIME IN HOURS	ADDED GLUCOSE HIGH CONCENTRATION SUSPENSIONS			ADDED GLUCOSE LOW CONCENTRATION SUSPENSIONS		
	K conc. M per L.	pH	Total glucose used	K conc. M per L.	pH	Total glucose used
			mg %			*mg %*
0	0.0943	7.18	0	0.0936	7.18	0
6	0.0892	7.00	160	0.0928	7.17	26
12	0.0850	6.86	410	0.0833	7.00	106
24	0.0840	6.73	705	0.0655	6.80	262
36	0.0770	6.65	863	0.0653	6.70	396
48	0.0600	6.75	1050	0.0865	6.79	545

possible significance of the terminal increase in K content of the cells suspended in low concentrations of glucose is treated in the discussion.

A third type of experiment was devised to circumvent the difficulty of maintaining pH constant. Ten milliliter portions

of rabbit blood were placed in 200 ml of 1% NaCl-phosphate buffer mixtures (see Methods). Two suspensions were prepared at pH 7.5, two at pH 7.0 and two at pH 6.5. To one of the suspensions at each pH glucose was added to bring the concentration in the suspension of 400 mg %. The other suspension at each pH contained no added glucose and analysis showed the glucose concentration in these suspensions to be less than 10 mg %. Figure 3 shows the loss of K from the cells of the 6 suspensions up to 48 hours. The graph reveals that the most rapid loss of K occurred in the suspension at

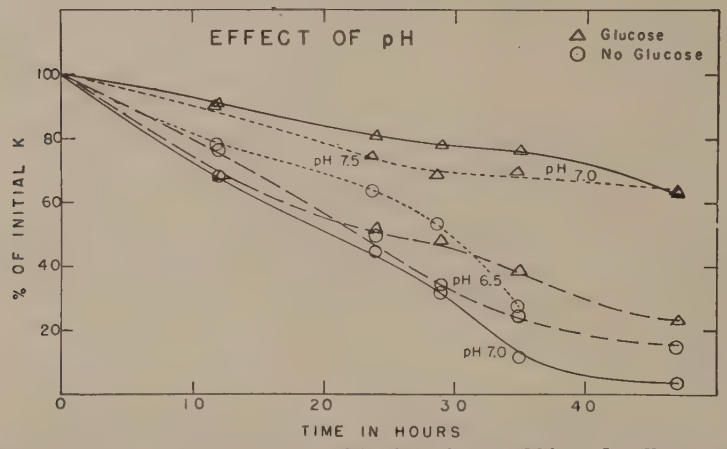

Fig. 3 Plot of potassium loss with time from rabbit red cells exposed to *"added-glucose"* and *"no-glucose"* suspensions at each of three pH values.

pH 7.0 containing no added glucose, while the least rapid loss of K occurred in the suspension at pH 7.0 containing 400 mg % glucose. At pH 7.5 differences of a smaller magnitude were noted between the cells in the *"added-glucose"* suspension and the *"no-glucose"* suspension than at pH 7.0. At pH 6.5 little difference in the rate of K loss was observed whether the suspension had added glucose or no glucose.

The magnitude of the differences in K loss between the *"added-glucose"* and the *"no-glucose"* suspensions at each pH studied is plotted in figure 4. These results indicate a generally unrecognized fact in this field, namely, that any interpreta-

tion of the effect of glucose on K retention in red cells must consider the important influence that pH has.

Table 3 shows the changes in pH and the glucose concentrations in each of the 6 suspensions during the period of observation.

As can be seen from table 3 the two suspensions whose initial pH value was 6.6, varied from 6.6 to 6.4; the two suspensions at 7.1 varied from 7.1 to 6.9; and the two suspensions

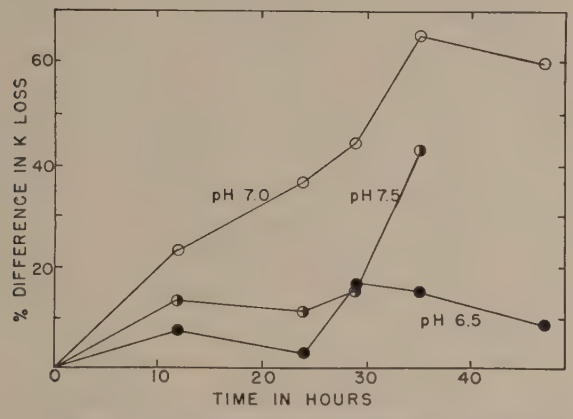

Fig. 4 Plot of the per cent difference in potassium loss with time from rabbit red cells exposed to *"added-glucose"* and *"no-glucose,"* at three pH values.

at 7.6 varied from 7.6 to 7.2. This last variation, which is the largest observed, may also account for the rapid upswing of the intermediate curve in figure 4 after 29 hours.

While the amount of glucose utilized appears to be lower than that shown in table 2, it must be remembered that the cell suspensions represented in table 3 contain only one-twentieth the number of red cells compared with the suspension recorded in table 2. Calculation shows that the glucose utilization per red cell is similar in the two experiments.

DISCUSSION

While extensive studies of cation movements in red cells were made by Ashby ('24) and by Kerr ('26 and '29), Mai-

zels ('35) appears to have been the first to call attention to the importance of maintaining the pH constant during the study of such movements. He also used glucose as a non-electrolyte suspension medium and observed that the amount of K loss was decreased by addition of traces of NaCl and KCl. It is very probable that the explanation of these results

TABLE 3

TIME IN HOURS	pH	*"No-glucose"* SUSPENSIONS	*"Added-glucose"* SUSPENSIONS
		mg % glucose (?)[2]	*mg % glucose*
0	6.6	5	425
	7.1	9	400
	7.6	10	395
12	6.5	2.5	413
	6.9	13	403
	7.3	4	365
24	6.5	2.5	375
	7.0	4	405
	7.4	4	370
29.5	6.5	1	400
	7.0	0	365
	7.3	3	350
35	6.6	0	380
	7.0	0	360
	7.3	2	355
47	6.6	0.5	375
	7.0	1.5	355
	7.2		350

is to be found in the large shifts of water that occur between a red cell and a non-electrolyte environment (Jacobs, Parpart and Corson, '37).

Davson ('40) has studied the uptake of K in the normally low K containing cat erythrocytes. He observed a more rapid penetration of both K and Na with increases in the alkalinity of the medium. His experiments covered a pH range from

[2] See Green and Parpart ('53).

5.8 to 7.9 and were performed chiefly in an isotonic KCl environment. Danowski ('41) working with human blood at 37°C. observed that additions of glucose at intervals after the beginning of incubation prevented the loss of K into the serum. He neither observed nor controlled pH change in the serum. He noted that the beginning of K loss coincided with the completion of glycolysis and was accompanied by an uptake of water by the cells. This swelling is probably associated with a decrease in pH of the system (Jacobs and Parpart, '31). Harris ('41) performed experiments similar to those of Danowski using washed human cells suspended in buffered saline. He added glucose to his saline solutions and determined the pH of the medium at the end of his experiments as well as the rate of glucose utilization. He confirmed Danowski's observation that K loss was retarded by the presence of glucose in the medium. Both of these investigators associated the retention of K with glycloysis.

Our results, shown in figure 1, bear out the findings of Danowski and Harris. However, that the pH difference between red cell suspensions with and without glucose cannot be ignored is emphasized by the data presented in both figures 2 and 3. Table 2 also reveals that under the conditions of the experiment reported, the utilization of glucose did not diminish when the pH fell from 7.18 to 6.70. Contrary to the findings of Danowski, we found in the experiment reported in figure 1 that the red cell volumes increased in the glucose suspensions and were reduced slightly in the absence of glucose (Jacobs and Parpart, '31).

In the data reported in figure 2 and table 2, no satisfactory explanation is available for the terminal leveling off and increase in potassium in cells maintained in a low glucose environment. This leveling off of potassium loss and slight uptake has been obtained in other experiments. The matter is being further investigated but tentatively it is suggested that the failure of the cells in the high glucose environment to maintain their potassium may indicate an exhaustion of some cellular material still in supply in the less actively gly-

colyzing red cell suspensions. The important point of the experiment for the present is that, when parallel, falling pH gradients are maintained in red cell suspensions, glucose utilization or concentration is shown to be not the only factors of importance in the potassium retention in the cells.

Figure 4 summarizes the results presented in figure 3 and shows the marked effect of hydrogen ions in accelerating K loss in the presence or absence of glucose. If, as the results of table 2 indicate, glucose utilization is not impaired within the pH range studied, it is difficult to attribute the results obtained in figure 3 to interference with glycolysis. These data are more in agreement with the idea that the character of the plasma membrane and hence its K permeability are modified by hydrogen ions or possibly by the hydrogen ion concentration gradient across the membrane (e.g. associated with lactic acid production). It is recognized that the K loss shown for pH 7.0 in figure 3 may involve additional factors. However, subsequent experiments at pH 6.9 indicated that a difference as great as 25% in K loss after 45 hours, between cells in suspensions with or without glucose were not observed. These latter findings are in agreement with the idea expressed above.

Granting that K loss from rabbit red cells is in a large measure conditioned by the pH of the external medium, the fact remains that the greater K retention, has, in our experiments, consistently been found in the medium containing glucose. Both Danowski ('41) and Harris ('41) have suggested from their experimental work that glycolysis is associated with this K retention. Roberts, Roberts and Cowie ('49) reported evidence that K retention in *E. coli* is associated with the phosphorylation of glucose. This mechanism has not been demonstrated to be effective in red cells but could account for the results observed by us. However, the differential pH effects reported in figure 3 strongly suggest that other factors may be involved. For example, an optimum pH gradient from cell interior to cell exterior, maintained by glycolysis may be essential for K retention; at low external pH values this gradi-

ent may be too small and at high values too steep to permit K retention.

SUMMARY

The rate of potassium loss from rabbit red cells maintained in sodium chloride-phosphate buffered solutions at various pH and glucose concentration levels was measured for periods of 48 hours at 35°C.

By replacing rabbit serum with equal amounts of glucose enriched, sodium phosphate buffered solutions and observing glucose utilization, pH change and potassium loss, it was found that the pH decreased from 7.18 to 6.75 in 48 hours. With a twofold difference in glucose utilization in different suspensions that showed the same pH gradients, the rates of potassium loss approximately paralleled each other.

Suspensions of cells in a high glucose environment were compared with those in a no glucose environment at pH 6.5, 7.0 and 7.5 for periods up to 48 hours. It was found that the most rapid loss of potassium occurred in cells in the no glucose environment at pH 7.0 and the least rapid in the high glucose environment at pH 7.0. At pH 6.5 there was little difference in the rates of loss of potassium from cells in high or no glucose. At pH 7.5 the rates of loss of potassium were intermediate to those observed at pH 6.5 and 7.0.

The significance of these findings was discussed and the importance of the hydrogen ion concentration and possibly a hydrogen ion concentration gradient was emphasized as a factor in the retention of potassium in red cells.

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THE EFFECT OF METABOLIC POISONS ON POTASSIUM LOSS FROM RABBIT RED CELLS

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FOUR FIGURES

A number of investigators have studied the action of such metabolic poisons as NaF, IAA, NaN_3 and NaCN on mammalian erythrocytes. Wilbrandt ('37) observed that NaF and IAA increased the osmotic resistance of red cells. In later work ('38, '39) he came to the conclusion that the action of these two compounds was primarily on the cell surface and only indirectly on glycolysis. Davson and Danielli ('38) found negligible potassium losses from rabbit red cells exposed for short time periods to NaF and NaCN. Davson ('41) subsequently obtained appreciable K losses from such cells exposed to NaF for longer times. He interpreted his results to mean that a metabolite was formed from the action of the poison which rendered a cell surface permeable to K. Harris ('41) and Danowski ('41), both working with human red cells, formed the opinion from NaF action on K loss that inhibition of glycolysis, with reduction of energy for maintenance of the K gradient, accounted for the loss. Ponder ('49) has recently examined the effect of NaF, IAA and NaCN on human red cells without arriving at definite conclusions although he suggests the possibility of injury effects. Flynn and Maizels ('50) and Maizels ('51) report evidence which they inter-

[1] This work has been supported in part by a grant from the John and Mary R. Markle Foundation and the Higgins Fund of Princeton University.

pret to mean that the control of cations in red cells is clearly dependent upon glycolysis.

The object of the present paper is to report the results of experiments in which the effects of metabolic poisons on red cells are examined, particularly as influenced by glucose concentrations.

METHODS

Fresh rabbit blood, obtained by heart puncture, was defibrinated and handled under sterile conditions throughout the experiments described. The cells were washed two to three times in isotonic NaCl solutions which were buffered with 0.033 M phosphate. (The NaCl-PO_4 solution was prepared by adding 150 ml of 0.11 M phosphate to 350 ml of 1% NaCl.) This amount of washing lowered the content of reducing compounds in the suspensions to about 15% of that in whole blood. Ten milliliter aliquots of the washed cells were placed in glass-stoppered bottles to each of which was added 200 ml of the 1% NaCl-PO_4 solution at pH 7.0. Usually these cell suspensions were paired, one member of the pair receiving glucose in amount sufficient to bring the suspension concentration up to 400 mg %; the other member of the pair received no glucose. To such paired cell suspensions were added metabolic inhibitors. The following inhibitors were used in these studies: 0.05 M sodium fluoride (NaF), 0.0005 M sodium azide (NaN_3), 0.0003 M iodoacetic acid (IAA) and 0.0001 M p-chlormercuricbenzoic acid (PCMB). The cell suspensions were incubated at 37°C. for periods up to 50 hours. During this time the cells were mechanically agitated by rolling on a Ball Mill rolling bed at intervals of 15 minutes "on," 15 minutes "off." During the incubation period, samples of the suspensions were taken at intervals usually of 6 to 12 hours. The cells from such samples were packed by air turbine centrifugation (10 minutes at about 20,000 G, Parpart and Green, '51), and the supernatant and top-most layers of cells removed. Measured amounts of the remaining cells were placed in distilled water for flame photometric analysis of the

K and Na contents by means of the Perkin-Elmer flame photometer. The cell volumes were determined spectrophotometrically by a measurement of the hemoglobin concentration. The analyses were corrected to a standard cell volume taken as the initial volume of the washed cells. In experiments in which glucose was studied, measurement of this substance was made by a modification of the method of Hoffman ('37).

RESULTS

1. The effect of iodacetic acid (IAA) upon potassium loss from rabbit red cells in the presence and absence of added glucose

Figure 1 shows the loss of K with time from cells suspended in an environment to which glucose was added (i.e. one containing about 400 mg % glucose) and from cells suspended in an environment to which no glucose was added. These environments are hereafter designated as *"added-glucose"* and *"no-glucose,"* respectively. In this particular experiment the initial glucose concentration of the cells represented by the white circles was 320 mg % ; that by the black circles was 16 mg % ; that of the white triangles was 348 mg % and that of the black triangles was 15 mg %. The cells added to the *"no-glucose"* environment had, initially, a concentration of reducing substances equivalent to the 15 and 16 mg % "glucose" given above. Data presented in figure 3 and discussed further along indicate that these reducing substances are probably not glucose.

It will be noted that the rate of loss of K from red cells in a *"no-glucose"* environment is identical during the first 20 hours, with that of cells in an *"added-glucose"* environment. After this time the *"added-glucose"* suspension lost K more slowly than did the *"no-glucose"* suspension (Parpart and Green, '53).

The results were strikingly different for the IAA treated cells as shown by the two lower curves of figure 1. The cells of both *"added-glucose"* and *"no-glucose"* suspensions were ex-

posed to 0.0003 M IAA. It is apparent that the cells in both of these suspensions lost K at essentially the same rate without regard to the glucose concentration. It is also to be noted that the rate at which the IAA treated cells lost K was considerably greater than the non-IAA treated cells. This fact indicates at least one and possibly the primary action of the IAA is on the plasma membrane of the cell, altering it in such a manner that the rate of loss of K from the cell interior is greater than in suspensions not containing the IAA.

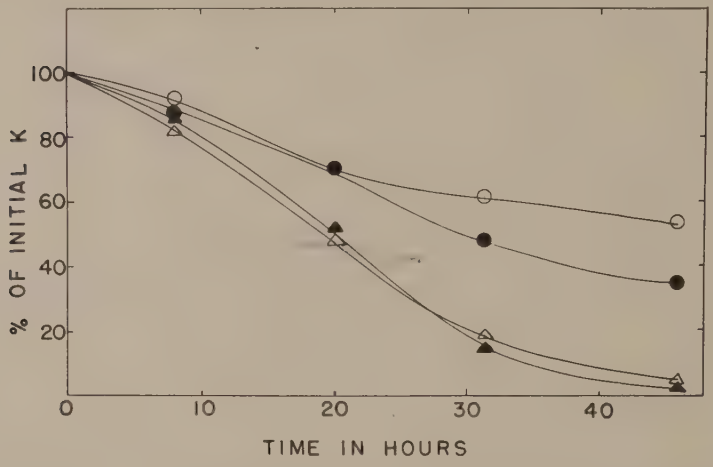

Fig. 1 Plot of K loss from rabbit red cells with time when cells are exposed to IAA. ○ = *"added-glucose"* control; ● = *"no-glucose"* control; △ = *"added-glucose"* with IAA; ▲ = *"no-glucose"* with IAA.

To obtain additional information on the action of IAA on red cell K loss an experiment was performed in which washed cells containing minimal amounts of reducing substances were incubated at 37°C. and pH 6.8. IAA was added to one suspension at zero time; to another at 6 hours and to a third at 21 hours. The cell K loss in all suspensions from the beginning of incubation was followed. Curves of the losses with time are shown in figure 2. These curves show that there is little or no "latency" in the acceleration of K loss from cells treated with IAA; they also show that after the addition of

IAA whether at 6 hours or 21 hours the accelerated K loss reaches that of the cells exposed to IAA at zero time after a delay time equivalent to the initial delay time, namely, 15 hours. Worth noting also is the fact that after 34 hours of incubation the loss of K is at essentially the same rate in all three suspensions containing the IAA.

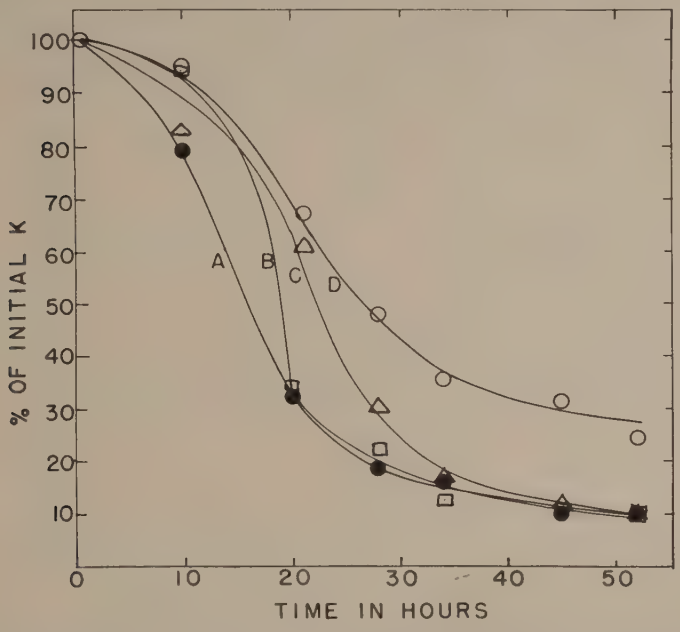

Fig. 2 Plot of K loss from rabbit red cells with time when cell suspensions are exposed to IAA at several intervals of time, *"no-glucose."* ● = IAA added at 0 hours; □ = IAA added at 6 hours; △ = IAA added at 21 hours; ○ = no IAA added.

During the first 45 hours of incubation the "glucose" concentrations of the suspensions were followed. These are shown in figure 3. The striking thing about the curves in this graph is the relatively uniform rate of disappearance of reducing substances in the suspensions whether they contain IAA or not. This must mean that whatever reducing substances remained in the cell after washing, are not altered in their rate of disappearance by the inhibitory action of the

IAA. The rate of loss of K from these red cells is shown in figure 2. As a consequence of this experiment it is difficult to understand how the IAA could be poisoning those systems which other workers have claimed are providing energy for the maintenance of the K within the cells. A more probable interpretation of the results shown in figure 2 is that the IAA is altering the plasma membrane of the cells in such a way as to increase the rate of K loss.

This interpretation is supported by our finding that acetic acid alone in the same concentration as in the IAA does not

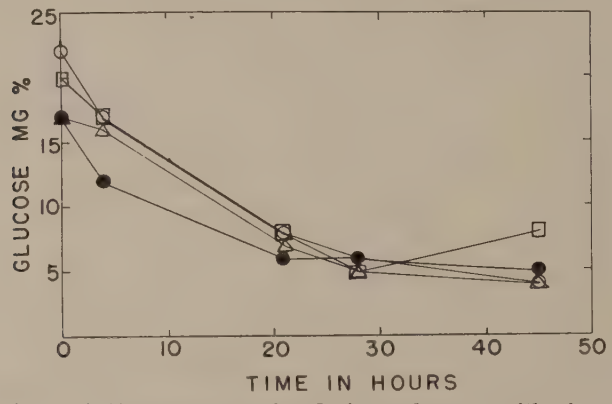

Fig. 3 Plot of disappearance of reducing substance with time in the cell suspensions shown in figure 2. ○ = no IAA; □ = IAA added at 21 hours; △ = IAA added at 6 hours; ● = IAA added at 0 hours.

have the same effect, nor does the iodine acting alone have this effect. Furthermore, the dilution of the cells by the buffering fluid was sufficiently great in this experiment to prevent any pH change during its course so that a pH effect is not being studied (Parpart and Green, '53).

2. The effect of the metabolic inhibitors sodium fluoride, sodium azide and p-chlormercuro-benzoic acid upon potassium loss from rabbit red cells

Additional inhibitors have been studied. Figure 4 shows the results obtained when rabbit red cells are incubated at pH 6.9

and 37°C. for periods up to 46 hours in the presence of various inhibitors other than IAA. Of the three inhibitors studied, PCMB had the most drastic effect in accelerating K loss. Actually only one analysis was made of PCMB treated cells and the times shown are those which elapsed after the addition of the inhibitor until the cells were prepared for analysis, a period between 30 and 45 minutes. However, during this period 40 to 60% of the K in the cells was lost. Although the concentration of PCMB used was 0.001 M it is possible that

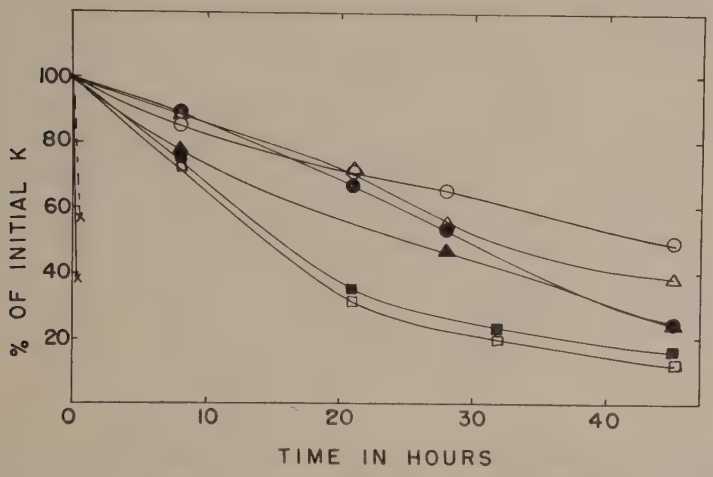

Fig. 4 Plot of K loss from red cells with time when exposed to NaN_3, PCMB and NaF. ○ = *"added-glucose"*; ● = *"no-glucose"* control; X = PCMB with or without glucose; △ = glucose + NaN_3; ▲ = NaN_3 without glucose; □ = glucose + NaF; ■ = NaF without glucose.

sufficient free Hg was present in the preparation to account for its extreme action. The NaF (marked by squares) shows an effect resembling that of IAA. Cells in an *"added-glucose"* environment (white squares) or *"no-glucose"* environment (black squares) lost K at essentially the same rate in the presence of NaF (0.05 M). Both rates were faster than the control rate for cells in a *"no-glucose"* environment (black circles). An interesting feature of the NaF curves is that when they are compared with the IAA curves on the same scale

(fig. 1) they reveal differences in shape and hence differences in rate of K loss. This feature favors the idea of surface alteration more than that of inhibition of glycolytic mechanisms. Of the inhibitors studied NaN_3 (0.0005 M) is the only one which did not exhibit the same effect on K loss from the cells whether in a *"no-glucose"* or an *"added-glucose"* environment. Its effect was not greatly different from that of the controls.

Table 1 shows the time in hours to 40% loss of the initial K value of cells in the presence of various inhibitors and enables one to compare the relative effectiveness of these inhibitors.

TABLE 1

Time in hours to 40% loss of K

COMPOUND	CELLS WITH GLUCOSE	CELLS WITHOUT GLUCOSE
	hours	*hours*
None	34	25
NaN_3	26	19
IAA	16	17
NaF	13	14
PCMB	0.3	0.3

DISCUSSION

While the results in figures 1 and 4 reveal that the rate of loss of K from rabbit red cells is unaffected by the absence or presence of glucose in the environment during the first 20 hours, nevertheless, exposure beyond this time does result in a slower rate of loss of K from those cells in the presence of glucose. This latter finding has been reported before for a variety of experimental conditions (Harris, '41: Ponder, '49; Parpart et al., '47; Maizels, '51). Since the work of Harris, the view has been widely held that energy derived from glucose break-down by way of glycolysis is utilized in some manner by the cell in maintaining high potassium and low sodium intracellular concentrations. Support for this view rests chiefly on the type of finding made by Wilbrandt ('37) and Harris ('41) and confirmed by others that

the metabolic inhibitors IAA and NaF, when added to red cell suspensions accelerate K loss. Biochemical work (see Lardy, '49) has shown that glycolytic enzymes are interfered with by these compounds and consequently their effect on red cells has been most frequently interpreted (see Ponder, '48) as an interference with the release of glycolytic energy. Without glycolytic energy those mechanisms in the cell which maintain the cation gradients, it has been presumed, would become ineffective and K would be lost to the external environment at an accelerated rate.

Figure 1 shows that for the first 20 hours of the experiment the loss of K from the cells is the same whether in an *"added-glucose"* or a *"no-glucose"* environment. After this time those cells in an environment without glucose lose K more rapidly than those in the *"added-glucose"* environment. However, the IAA treated cells whether with or without glucose have lost about half of their K by 20 hours and by 48 hours have lost over 90% of it. This striking parallel in the action of IAA on cells with and without glucose is what would be expected if the IAA were acting as a lytic agent (Parpart and Green, '51). Since the potential sources of energy within the *"added-glucose"* cell suspension are so much greater than in the *"no-glucose"* suspension, it would be expected that an inhibitor acting on glycolytic enzymes only would have a lesser effect on the *"added-glucose"* cell. The failure of this to occur as shown by figure 1 is substantial evidence in favor of the IAA acting as a surface disorienting agent.

This view is further substantiated by the results shown in figure 2. In this graph we see the effects of adding IAA to *"no-glucose"* cell suspensions at the beginning of the experiment, after 6 hours and after 21 hours. In figure 1 it was shown that after 20 hours the control cells without glucose begin to lose K more rapidly than control cells with glucose. If this indicates an exhaustion of glycolytic energy then one would suppose that the addition of IAA would have an appreciable effect on the acceleration of K loss. However, in figure 2 it is found that the addition of IAA at 21 hours accel-

erates K loss in the same manner as occurred upon the addition at the beginning of the experiment. Again this is the expected behavior of a compound altering the surface of the cell.

Additional support for the view that the chief action of IAA on red cells in accelerating K loss lies in its action on the surface of the cells is to be found in the curves shown in figure 3. Here it is seen that whatever reducing compounds remain in the cell after thorough washing, disappear at the same rate whether in the presence or absence of IAA. It is possible that reducing compounds are being shunted into a direct oxidative pathway (Dickens and Glock, '51) for the production of energy for K retention. If this is so it is difficult to understand why *"added-glucose"* and *"no-glucose"* cells lose K at the same rate when treated with IAA. IAA cannot, therefore, be acting solely or even primarily as an inhibitor of glycolysis in these experiments.

The results shown by figure 4 support to some extent the conclusions drawn from figures 1, 2 and 3. The behavior of NaF is very similar to that of IAA and it is thought to be producing an acceleration of K loss by altering the cell surface. PCMB was so lytic and acted so much more rapidly than the other inhibitors that we have assumed that the results we obtained were in large measure attributable to the presence of free mercury in the solution. The action of NaN_3 was very similar to the behavior of the control without glucose. It is not considered to have had any large surface effect.

It may be concluded that the results reported in this paper indicate that the action of IAA, NaF and PCMB is not confined to glycolysis alone in the red cell but that under the experimental conditions used by us, the surface disorienting action of these compounds would appear to overshadow any effect they may have on glycolysis insofar as the acceleration of K loss is involved. Other investigators of cation exchange in red cells have used IAA and NaF in approximately the same concentrations as here reported and it is considered likely that their results are a consequence of the prelytic ac-

tion of these compounds as well as their effect on glycolysis. There is no doubt that these inhibitors influence both the rate of glycolysis and the orientation of molecules in the plasma membrane. At present we believe the latter is the more important effect in relation to K retention. It should also be noted that studies on the action of the inhibitors such as IAA, NaF and PCMB on cells other than red cells may involve similar surface disorientation action. Thus physiological changes in cells observed in the presence of these inhibitors may not be solely or even primarily due to their action upon the metabolic cycles of the cell.

SUMMARY

Rabbit red cells were washed in phosphate buffered-isotonic NaCl solutions until the content of the reducing substances in the suspensions was decreased to about 15% of that in whole blood. Upon incubation with metabolic inhibitors these remaining reducing substances decreased at the same rate as in the absence of inhibitor.

Paired aliquots of these washed cell suspensions, to one of which 400 mg % glucose was added, were exposed to the action of 0.05 M NaF, or 0.0005 M NaN_3, or 0.0003 M IAA or 0.0001 M PCMB under sterile conditions at 36.8°C. for periods up to 48 hours. NaN_3 had no appreciable effect in accelerating K loss over that of the glucose controls. PCMB was found to be extremely lytic; the cells hemolyzed in about 30 minutes, and it greatly accelerated K loss over non-inhibitor controls.

The action of IAA and NaF was essentially similar; both accelerated the K loss from either *"added-glucose"* or *"no-glucose"* suspensions. In both cases the loss of K was greater than in control, no inhibitor, suspensions. This action of NaF and IAA is interpreted to mean that both of these substances in addition to attacking the enzymes of glycolysis, produce an alteration of the cell surface which renders it more permeable to K. It is emphasized that this prelytic action of

these inhibitors should be taken into account in the study of the action of inhibitors on the metabolism of cells and tissues.

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ON THE ULTRASTRUCTURE OF THE PLASMA MEMBRANE AS DETERMINED BY THE ELECTRON MICROSCOPE [1]

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THIRTY-FOUR FIGURES

INTRODUCTION

The erythrocyte plasma membrane has received comparatively intensive study for many years. Many of its physiological characteristics are now established, and much is known about its chemical composition (Jacobs, '30; Parpart and Dziemian, '40; Davson and Danielli, '43; Ponder, '48; and Parpart and Ballentine, '52). On the other hand, indirect evidence leading to an understanding of the orientation of membrane components at the molecular level is still inadequate while direct evidence is all but non-existent.

There have been essentially two recent theoretical approaches to the ultrastructure of the plasma membrane. After consideration of (1) the ready penetration of lipid soluble substances into erythrocytes, (2) the total lipid content of the erythrocyte (Gorter and Grendel, '25), (3) the calculations from electrical impedance measurements (Fricke, '25) and (4) the lowering of the interfacial tension relative to values found in living cells by proteins at the surface of a lipid phase, Harvey and Danielli ('38) proposed a paucimolecular

[1] A portion of this paper was reported at the Federation Meetings April 17, 1952 (Hoffman and Hillier, '52).

[2] This work was presented to the Biology Department of Princeton University in partial fulfillment for the degree of Doctor of Philosophy. This study was in part supported by the Eugene Higgins Fund of Princeton University.

layer hypothesis. This postulates a continuous bimolecular layer of lipid (radially oriented) bounded on both sides by tangentially oriented protein. Parpart and Ballentine ('52), on the basis of the differential binding of lipids to the ghost, the relative proportions of each per unit protein, and the hemolysis and permeability properties of the erythrocyte, proposed a protein-lipid lined pore theory. In this model the major portion of the lipid is oriented tangentially as is the protein.

The advent of the electron microscope in the late 1930's and the subsequent attainment of high resolution has provided the possibility of direct visualization of the ultrastructure of the plasma membrane. Furthermore, the red cell represents an ideal material for study since the physiological background which is very necessary in the interpretation of the electron micrographs is already extensive. The purpose of this investigation was to elucidate the fine structure of the plasma membrane, to relate this to the relevant physicochemical data available, and to evaluate the modern theories in the light of the present findings.

Historically, Wolpers ('41) was the first to examine red cells and their ghosts with an electron microscope. Though the intact unhemolyzed cell was too dense for penetration by electrons, he demonstrated that the ghost was suitable for direct study.

The work since then (Wolpers and Zwickau, '42; Angelo et al., '49; Lindemann, '49; Bessis, '50; Jung, '50; Zacek and Rosenberg, '50; Epstein et al., '51) has not yielded results which can be compared directly with those presented here, in part due to insufficient resolving power and in part due to inadequate control on the cleanliness of the specimen. There does not seem to have been a systematic evaluation of methods for the preparation of ghosts.

The study of intact cells is possible by the replica method as described by Hall ('50) or modifications of it; a method which was devised in the hope that hemoglobin contamination would be averted. The coarseness of the structure of the

replicating material is the inherent limitation of the procedure. The results obtained (Rebuck and Woods, '48; Bessis, '50; Rhian et al., '51; Latta, '52) have not been especially encouraging.

In none of the aforementioned work is there any demonstration of structures of the type and dimensions encountered in the present work. On the other hand many of these papers describe rather large structures which are now recognized as gross artifacts. The foundations of the present study are the consistent attainment of resolutions to the 20 A level, a careful application of the shadowing method of Williams and Wyckoff ('44) and a consideration of the problem of controls with respect to the preparative techniques.

MATERIALS AND METHODS

Two drops of whole blood were collected into 1 cm^3 of 0.17 m NaCl in which 4 mg of sodium citrate had been previously dissolved (a separate controlled experiment had indicated that there was no observable change in the fine scale morphology resulting from the use of the citrate). Blood from the following animals was used: normal human male (ages 20–30), rabbit, rat and groundhog. Finger puncture was employed on man, and cardiac puncture on the other mammals. The rat and groundhog were anaesthetized with ether prior to collection.

It was recognized at the outset that reproducibility could be achieved only if the ghosts were as free of hemoglobin and other contaminants as possible.[3] Consequently, by following

[3] The basic assumptions of the method described are that at the time of hemolysis (of the osmotic type) the surface of cell is slightly altered (cf. Ponder, '48) so that it becomes permeable to hemoglobin and cations and that hemolysis proceeds by diffusion of hemoglobin from the cell. The time available for hemolysis is limited since the cell again becomes impermeable to hemoglobin when the osmotic pressure is decreased by the loss of internal cations. Thus the time available is insufficient to permit all of the hemoglobin to diffuse out of the cell. This means that some hemoglobin will always remain in the cell after the initial hemolysis. On the other hand proportionate amounts of hemoglobin will leave the ghost if its osmotic pressure can be increased several times in succession.

the method outlined below intact, presumably Hb-free, ghosts are obtained. The principle involved takes advantage of the osmotic resistance curve of normal human erythrocytes where 0.03 m NaCl will produce 100% hemolysis. Thus it becomes possible to subject the cells to a series of osmotic shocks by washing successively in NaCl solutions of increasing hypotonicity.

The application of this method of successive hemolysis for the preparation of ghosts was the following: one drop of the diluted blood was washed 4 times with 20 cm^3 of 0.17 m NaCl. These washings reduced the serum protein content to less than one part in 10^{10}. (calculation shows this results in less than 200 plasma protein molecules per cell). The cells were then concentrated, centrifugally, and hemolyzed in a ratio of one part of cells to 400 parts of 0.03 m NaCl. This was repeated three times but with NaCl concentration decreased to one-half the former concentration with each wash. Hence, the ghosts were finally centrifuged down in 0.0037 m NaCl. These were resuspended in 0.0037 m NaCl and an aliquot of this was layered at the top of a flat-bottomed tube also containing 0.0037 m NaCl. At the bottom of this centrifuge tube there was a copper mesh screen (mesh size: 200 to the inch) with a thin (ca. 100 A thick) collodion membrane attached. The ghosts were then centrifuged on to the collodion mount. The screen with ghosts mounted in this fashion was then removed and washed repeatedly in redistilled water.

During the whole process of preparation only about one out of 10 ghosts expected appeared on the collodion. One explanation may be that the ghosts stick to the side of the glass centrifuge tube, for the wall became progressively non-wettable by water. The ghosts are known to be sticky at an acid pH (the pH of the NaCl solutions used above was ca. 5.5) and also to possess a hydrophobic surface (Mudd and Mudd, '26). The fact that there was a decrease in the number of ghosts (the number recovered is of the order of 2.5×10^6) could indicate a selection of the sample. However, some preliminary

experiments involving hemolysis directly on the collodion mount indicated that this is improbable.

All experimental treatments were carried out after the ghosts were attached to the collodion membrane but before drying. The exposure time to a particular agent varied between 5–15 minutes with the exception of one to two minutes for OsO_4. The solutions used were: the vapors of 2% OsO_4; 0.1% phosphotungstic acid; 10% formalin; 70% alcohol; 95% alcohol-chloroform (ratio of 3:1); 95% alcohol-chloroform-ether (ratio of 3:1:1). When desired the pH was controlled by the addition of a sufficient amount (ca. 0.0005 m) of Sorensen phosphate buffer to the experimental solutions. When any sequence of treatments was used the ghosts were washed between each step in redistilled water. After the final wash the mounted ghosts were then air dried (facilitated by drawing excess water off with filter paper) or frozen-dried by a new method to be described by Hillier ('53). The total lapse of time from the withdrawal of blood to the drying of the cells was always of the order of two to three hours. The time to the first hemolysis was always less than 40 minutes.

All centrifugations were carried out with an International centrifuge at 3500 $\times$ g for 5 minutes. The preparation and treatments of the ghosts were done at room temperature.

An RCA Model EMU electron microscope was used with a high resolution double objective. No aperture was used in the objective but the projector system was carefully diaphragmed to eliminate background due to wall scattering. The shadowing was accomplished by a modification of the Williams and Wyckoff ('44) technique in which a very high vacuum was obtained, and ca. 5 A of chromium metal was evaporated at an angle of 4:1.

It was absolutely necessary to have all glassware very clean. Thus routinely, the glassware was first washed with a commercial cleanser ("Bab-O") then rinsed 8–10 times in tap water followed by 8–10 rinses with distilled water and finally oven dried. Precautions were also taken to use water and reagents as free from contamination as possible. Consequently,

Pyrex redistilled water was used. All solutions were prepared immediately prior to using and were centrifuged before exposing the ghosts to them. A supernatant solution which gave a minimum of residue on drying was obtained in this way. Prior to the adoption of these precautions, the ghosts examined by electron microscopy were found to have a film covering them.

Twenty-nine individual blood samples yielding 315 electron microscope specimens were examined. A total of 1965 exposures were made. Usually 5 cells for each sample were photographed. These were chosen as being representative by visual observation of at least 50 cells. In order to check this method of sampling and also to have some reliable data on the variability within a sample the first 50 cells observed were photographed in the case of two samples. The distribution with respect to primary treatment of the ghosts was approximately one-third controls, one-third stained or fixed, and one-third exposed to lipid solvents.

The majority of the micrographs were taken at an electronic magnification of 15,600 (unless otherwise stated, this is the electronic magnification of all the micrographs appearing in this paper). The illustrations presented were selected to represent the most commonly occurring structure for any given treatment.

The structures which are of interest in this work lie at least an order of magnitude below the resolving limit of the light microscope in a field where criteria of normalcy have not yet been established and in a range where independent means of determination are non-existent. In such a situation the development of adequate controls becomes a major part of the research.

The basic control in each experiment in the present work was half of the screen which was cut from the specimen immediately preceding the experimental treatment. This part of the screen did not receive the treatment but was returned to and carried through all the subsequent processing with the experimental half. A second control, particularly against con-

tamination, was provided by including in each micrograph an edge of the cell and a portion of the collodion membrane. The latter could then be compared with the known picture of clean collodion, appropriate allowance being made for variations in focus. In cases where treated cells were frozen-dried the untreated portion of the specimen was also frozen-dried and became the control. Since in the frozen-dried specimens the cells remained spherical it was found possible to remove their tops and thus expose their inner surfaces by simply pressing the preparation against a cleaned flamed microscope slide and removing. The spherical cells prevent the collodion from coming in contact with the glass, while those parts of the cells which come in contact with the glass remain stuck to it.

RESULTS

In addition to the following text the illustrations should be considered as part of this section. Some duplication has been avoided by giving the details of the treatment and pointing out the essential features in each figure only in the legends. The text carries a discussion and correlation of the more important observations resulting in the presentation of a working hypothesis consistent with the data. Unless otherwise stated, the data, description and figures are for ghosts of human male.

Figure 1 is representative of the structure observed in untreated air-dried cells prepared by the method of successive hemolysis. The surface is covered with structures which have been given the name, "plaques." The plaque is characterized as being roughly a cylinder approximately 30 A thick with a diameter varying between 100 and 500 A depending on the sample, individual and species. They are arranged apparently at random in chain-like groupings or singly forming a single layer. Each cell membrane possesses a characteristic "texture."

The reproducibility of the technique was established by dividing a sample of blood into 4 aliquots and processing them

separately. The specimens thus obtained appeared identical. (See also plate 5.)

The stepwise removal of contamination from the ghost is illustrated in plate 2. Figure 4 represents the typical degree of cleanliness obtained after three stages of the successive hemolysis followed by simple mounting, washing and air drying. There was no apparent improvement beyond the 4th stage (cf. plate 3) if the cells were washed two more times in 0.0037 m NaCl before mounting. In the case illustrated in figure 5 the blood was not washed free of its plasma before hemolysis. It is to be noted that the character of the surface is similar to that of the cells given the thorough washing prior to hemolysis. The difference in plaque size is believed to be characteristic of the blood used in this experiment. This observation, in addition to considerations involving the size of the plasma protein molecules (Oncley, '50) relative to the thickness of the membrane make it unlikely that the ghost surface has any residual plasma adsorbed. Parpart (unpublished) has found that antibodies produced by the injection of well-washed human ghosts into rabbit will not form an antibody to the host plasma proteins, though hemolysins are formed. This is further evidence for concluding that plasma protein is not present on the ghost surface.

A careful study of high magnification unshadowed pictures (figs. 21, 22, 23) shows that there is a fibrous component in which the fibers have diameters of the order of 20 A. It is difficult to determine whether these fibers are part of an underlying membrane to which the plaques adhere or are part of the plaques themselves. In figure 25 where the plaques have been removed the presence of an underlying membrane is demonstrated and there is some evidence of a fibrous component. Figure 34 is a shadowed micrograph of the inside surface of the intact plasma membrane in which the fibrous component is more obvious, but it must be remembered that surface contaminants introduced during the shadowing process cause structures larger than the fibers themselves.

The thickness of the single membrane is 50 A ± 10 A and was calculated from the length of the shadow, and the known shadowing angle. Since the diameter of the fibers are of the order of 20 A and the plaques have a thickness of the order of 30 A (fig. 25) it appears improbable that a layer of hemoglobin could be adsorbed on the inner surfaces. The smallest dimension of hemoglobin is 34 A (Lemberg and Legge, '49) which would yield a membrane thicker than the observed result. However, the possibility that some hemoglobin may be present on both the cell and the collodion cannot be completely ruled out, since a really close approach to the clean collodion structure pictured in figure 2 was rarely reproduced in specimens with mounted ghosts.

Plates 3 and 4 show that treatment with PTA introduces a recognizable artifact into the surface structure such that the plaques assume a more discrete appearance perhaps caused by an asymmetric shrinkage of the plaques relative to the substrate. The total thickness of the membrane is increased to about 60 A when exposed to PTA. Comparison with the controls showed that while the change in plaque size is artificial it did not change their arrangement, nor did it confuse the determination of relative differences in plaque size among different samples. In view of these observations and since the PTA-treated specimens were generally much cleaner, providing greater clarity in the pictures, most of the critical comparisons were made with PTA-treated specimens.

In addition to the change in plaque structure, PTA also enhanced the fibrous component as seen in the unshadowed preparations (plates 7, 8). After first being exposed to PTA the membrane structures are not essentially altered by subsequent treatments with alcohol, alcohol-chloroform, formalin or osmium tetroxide. However, as will be shown below, if ether was added to alcohol-chloroform a change was produced. These observations suggest that PTA acts both as a fixative and as a stain (Hall et al., '45) for protein in a somewhat selective fashion. The action of PTA was the same at pH 7.4

and at pH 2.8 (plate 5). One per cent NaCl did not affect this result.

Osmium tetroxide yielded the same type of result as PTA on the shadowed specimens. In the unshadowed preparations, however, OsO_4 was inferior to PTA for the elucidation of fibers. The reason for this may be that osmium has no selective action on the materials in the plasma membrane.

One observation which was made in the course of this work was that the mean plaque size varied from one individual to the next by amounts much greater than the range of variation within numerous samples from a single individual. The extremes encountered in the range of samples examined in this work is illustrated in plate 3. In at least one case the mean plaque size in the blood of one individual underwent a sudden change after remaining constant for several months. Preliminary observations indicate that there are even greater variations in mean plaque size among species (plate 12).

Exposure of the ghost (cf. Parpart and Ballentine, '52) to lipid solvents, e.g. with alcohol-ether-chloroform, resulted in the removal of plaques from the cell membranes and in their appearance on the collodion membrane. The plaques thus removed appeared very similar to those on the unexposed ghosts. The possibility that the plaques observed on the collodion are identical with those on the intact ghost would suggest that they are insoluble in water and lipid solvents.

The plaque structure also appears in specimens which were frozen-dried (figs. 32 and 33) and furthermore, showed similar dependence on the nature of the treatment. In general, the plaques appeared slightly less discrete in the frozen-dried specimens (compare plate 3) than in the corresponding air-dried specimens. This is in accordance with the anticipated reduction of surface tension artifacts.

Since the frozen-dried ghosts remain spherical except for a flattened circular area which was in contact with the collodion membrane, it was often possible to rip off all the ghost except that in the contact area. This made it possible to shadow the *inner* surface of the membrane. While somewhat differ-

ent from the outside, the appearance of the inner surface (fig. 34) was not as different as would have been expected if the ghost had not been dried, and if the ghost had not been in contact with the collodion membrane. Both of these conditions would lead to wrapping of the fibers of the inner lining closely around the inside surfaces of the plaques thus distorting the natural arrangement of the fibers.

Stereoscopic views of the frozen-dried preparations showed the interior space of the cells to be free of structure. This is good presumptive evidence that an internal network which could account for the biconcave shape does not exist. The final proof requires a frozen-dried biconcave ghost to be devoid of internal structure. This has not been accomplished, as yet.

Thus, two levels of organization have been demonstrated in the ghost: (1) fibers and (2) plaques. The fibers are seen to be oriented tangential to the surface. The experimental results suggest that the fibers comprise an inner lining to the plasma membrane. The plaques appear to be situated exterior to the fibrous lining and cover the major part of the exterior surface. There is evidence that the plaques possibly also have a fibrous structure (plate 11).

It is possible to arrange the treatments given to the ghosts according to the degree of change that they may be expected to produce in the structure. It then becomes apparent that the plaques observed are very likely representative of a similar structure present in the intact wet ghost.

From these considerations, a model can be constructed which is compatible with all of the results obtained so far in these studies with the electron microscope. Since the plaques can be removed by lipid solvents but do not appear to be greatly changed by them, lipids must, in some way, be involved in the attachment of the plaques to an underlying component which is probably a fibrous membrane. In the micrographs (plates 9 and 10) of the ghosts exposed to lipid solvents it must be realized that the entire specimen consists of the collodion membrane on which is superimposed the double layer of ghost

membrane. The plaques on the upper surface are removed while those between the lining and the collodion membrane are trapped in place. In addition there is probably some material trapped inside the cell. It has not been possible as yet to determine the degree of orientation of the fibers in the lining since the situation is confused by the overlapping of the two layers and by the resolution limit of the instrument.

DISCUSSION

Arguments concerning the structural orientation of molecules and groups of molecules in the plasma membrane of the intact red cell as determined on the ghost are presented with a full realization of the limitations of the method used. The electron microscope approach to this problem does, however, give the best visual picture that is obtainable at the moment of the physical arrangement of membrane components. While artifacts have been eliminated wherever possible, relating the structures observed in the dried ghost to the original intact erythrocyte remains a very real problem.

There is a certain amount of information available concerning the physiology of the ghost which contributes to the belief that the plasma membrane of the intact cell and the ghost are not too dissimilar in structural organization. What facts are known qualitatively, if not entirely quantitatively, indicate that the ghost exhibits many characteristics of the intact erythrocyte (Parpart and Ballentine, '52). They display the disc-sphere transformation (with constant surface area); return to the original volume after lytic hemolysis or osmotic hemolysis when, in the latter case, the salt concentration is returned to isotonic. The ghosts undergo volume changes at measurable rates (Teorell, '52) and while they momentarily behave as perfect osmometers they are not as stable at equilibrium as the intact cell; and they are impermeable to hemoglobin. Jacobs ('50) has shown that the plasma membrane is very likely identifiable with the permeability barrier of the intact cell.

A working hypothesis assumes that the ghost is not radically different from the plasma membrane of the original red cell. Since the electron micrographs show the same basic structures to be present independent of the various treatments, it becomes a reasonable assumption that the relationships illustrated by the model previously described can be applied to the intact ghost. In this manner the ultrastructure of the ghost can be useful in the evaluation of previous theories which were based on indirect evidence of the structure of the plasma membrane.

Moskowitz et al. ('50) isolated from ghosts a lipoprotein which they named "elinin" and which was characterized by their method of isolation. Aqueous solutions of the elinin were obtained by first extracting frozen-dried stroma with dry ether followed by replacement of the ether by water. They found that all of the activity of Rh, A and B factors resided with this elinin fraction. What remained following extraction was essentially hemoglobin and a material they called S-protein. Electron micrographs (Dandliker et al., '50) showed elinin to be rods ranging in length from 2500 to 10,000 A with the narrowest width of the order of 125 A. Chains of units 125 A in diameter could give rise to the rods.

Moskowitz and Calvin ('52) were thus able to construct a model of the plasma membrane. They proposed elinin to be linked together via the ether-extractable lipids to comprise the main (fibrillar) framework of the cell (called "stromin"). Associated with this would be the S-protein and hemoglobin. This model is in essential agreement with Wolpers' ('41) findings referred to before in the legend of figure 20.

It seems highly probable that the plaques are identical with the elinin fraction discussed above. First, the dimensions are quite similar especially if elinin is considered to be linearly arranged plaques. Second, it was shown that ether was necessary for the removal of plaques from the ghost. The evidence presented in figure 25, on ghosts which have been exposed to lipid solvents and then returned to water before drying, strongly suggests that the plaques removed to the col-

lodion are probably the same as elinin. This is particularly noticeable in the chains of plaques which are comparable to the electron micrographs of Dandliker et al. ('50). Further work along this line is being carried out. However, our model is not compatible with that of Moskowitz and Calvin ('52), the main difference being in the orientation of the ether-extractable lipids.

The fact that in the present work, it is the ether which is responsible for the removal of plaques from the underlying fibers permits further insight into the intimate structure of the plasma membrane. Parpart and Ballentine ('52) found that all of the cholesterol and 50% of the cephalins of ghosts are removed by dry ether. Thus the attachment of the plaque to the fibrous component could be in the form of a bimolecular leaflet in the sense of the hypothesis of Harvey and Danielli ('38). The hydrophilic ends of the phospholipid would be attached to the protein giving rise to: fibrous protein-lipid-lipid-plaque, from inside to out. This provides a radial orientation to a portion of the lipid which may be sufficient to satisfy the birefringence demands of Schmitt et al. ('36). The positive birefringence observed in this laboratory of the whole cell and of the ghost is indicative of a high degree of tangential orientation of the surface molecules. However, the number of radially arranged lipid molecules necessary to hold the plaques to the fibrous component is probably not large and permits the rest to be tangentially oriented as described below.

The remainder of the phospholipids could be associated with the plaques in a manner described by Parpart and Ballentine ('52). Thus the pores which are presumed to be between the plaques would be lined by lipid molecules. Calculation of the number of plaques per cell is of the order of 10^4 to 10^5. The number of pores is approximately equal to or double the number of plaques depending upon the arrangement chosen. It is an interesting fact that Parpart and Ballentine ('52) on physiological grounds arrive at the same order of magnitude for the number of pores present per cell.

Furthermore, pore size depends upon and varies with plaque size. The pores have minimal size for hexagonal close-packing of the plaques. For plaques 200 A in diameter the minimal pore size is then calculated to be ca. 35 A. This is not quite large enough for hemoglobin to diffuse through.

It is well known that pore size is not the only factor determining the permeability of a cell (Jacobs, '50), but it could play an important role in explaining species differences in permeability. Plate 12 is suggestive of this difference.

The significance of the variation in plaque size in humans is still obscure. This may be due to differences in (1) blood groups (one experiment designed to test this gave equivocal results), (2) age of the cells, (3) physiological state of the individual at the time of withdrawal of the blood. It would also be interesting to know to what extent these variations in plaque size are physiologically important.

CONCLUSION

A direct study of the plasma membrane has been made using electron microscope techniques. The use of a wide variety of techniques has made it possible to achieve some understanding of the artifacts involved and to devise a model of the structure which provides the possibility of reconciling two formerly divergent theories. The heterogeneous structure shown in this work could not have been demonstrated by any indirect technique. If adequately confirmed it should serve as a realistic framework with which the results of all other experiments must be compatible.

The authors wish to acknowledge their indebtedness to Prof. A. K. Parpart for his many enlightening and stimulating discussions during the course of this work.

SUMMARY

1. A new technique, designated as the method of successive hemolysis, has been devised for the preparation of essentially hemoglobin-free ghosts. This consists of consecutive rehe-

molysis in solutions of decreasing tonicity. The ghosts are then mounted on a collodion film and treated experimentally. The ghosts are found to be essentially free from contamination.

2. The control is the air or frozen-dried untreated specimen. The membrane appears to consist of a mosaic of structures which have been labeled "plaques." The plaque is characterized as a cylinder ca. 30 A in height and on the average ca. 200 A in diameter.

3. Phosphotungstic acid (PTA) causes the plaques to become more discrete. This is not influenced by pH or tonicity. PTA has been found to act as a fixative since upon subsequent exposure to formalin, alcohol, alcohol-chloroform, there is no change in the appearance of plaques. The order of treatment has also been found to be important.

4. In the unshadowed preparations, a fibrous component has been demonstrated. PTA increases the relative density of the fibers. The diameter of the fibers is of the order of 20 A. Their length varies but in general is around 200 A.

5. Osmium tetroxide is similar to PTA in acting on the plaques but is inferior to PTA in staining the fibers.

6. Alcohol and formalin by themselves alter the entire surface abnormally. This has been discussed in the light of former work.

7. When the ghosts are extracted with an alcohol-ether-chloroform mixture the plaques are separated from the surface thus fractionating the plasma membrane into two components: plaques and fibers. Ether is found responsible for this removal.

8. The thickness of the membrane is estimated to be 50 A.

9. Frozen-dried ghosts viewed stereoscopically appear to be free of internal structure.

10. A variation in plaque size has been found in individual samples of the same blood, among samples from different individuals and among species. The significance of the differences is discussed but is not yet clear.

11. A "plaque theory" of the ultrastructure of the plasma membrane is presented which is based on and is compatible with all of the results. The resulting model consists of plaques situated on the outside of a fibrous network joined together by ether extractable lipids. The areas between the plaques are considered to be the pores. This means that pore size will vary with plaque size and arrangement.

12. The implications of the plaque theory are discussed in relation to former hypotheses. The plaque theory offers the possibility of unifying the pore theory and the paucimolecular theory.

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PLATES

The magnification in all micrographs is 75,000 × unless otherwise stated and the micrographs are so oriented that the shadow-casting direction is downwards.

PLATE 1

EXPLANATION OF FIGURES

1 An electron micrograph of a portion of an air-dried ghost, shadowed. This is typical of the untreated control specimens. Noteworthy here is the plaque structure which covers the surface of the ghost. The distribution is apparently random with the size varying only slightly over the entire surface. At the top is seen the edge of the cell and a portion of the supporting collodion film. This can be compared with figure 2 for an indication of the amount of contamination present. Large masses such as the one appearing in this micrograph have been observed frequently on all specimens. Stereoscopic views of frozen-dried preparations have indicated many of these lie within the ghosts (6272c).

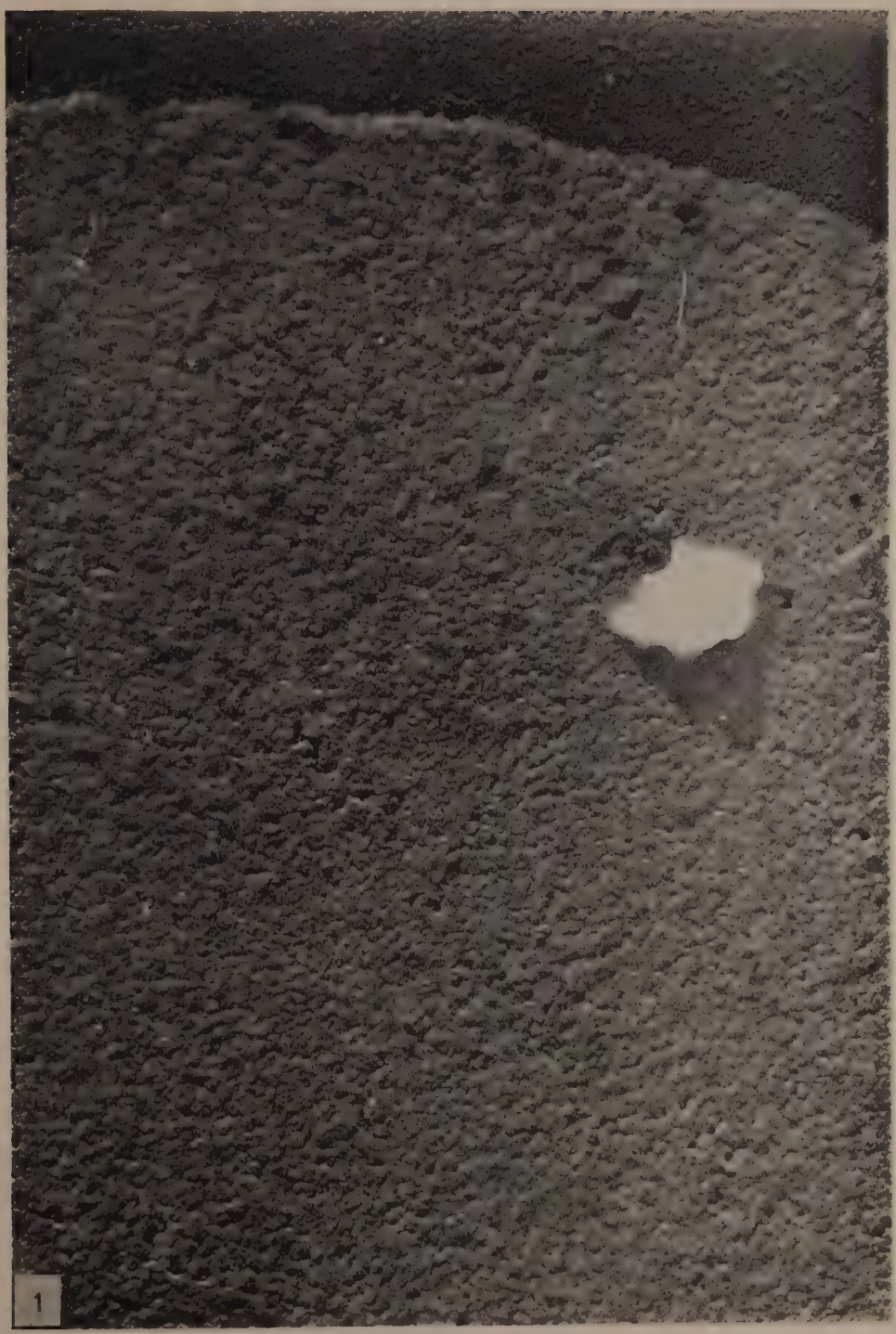
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PLATE 2

EXPLANATION OF FIGURES

2 Untreated collodion. Shadowed. This membrane was formed on water from 2% parlodion in amyl acetate, picked up on a wire mesh, dried in air and subsequently shadowed. This represents the structure of normal, clean collodion (5968).

3 Air dried control. Shadowed. Prepared by hemolyzing washed cells in 0.03 m NaCl and centrifuging directly onto a collodion mount. Note that the edge of the ghost is not definite. There appears to be a layer of extraneous material over both ghost and collodion. This is thought to be mainly hemoglobin (6301b).

4 Same as figure 3 except this ghost had been carried through three stages (therefore, through three washes) of the successive hemolysis technique. The collodion is still not as free of contamination as in figure 1, but more detail in the surface structure can now be discerned (6316e).

5 Air dried control. Shadowed. This cell was not washed of its plasma, but taken completely through the standard method of successive hemolysis. Of interest is that this ghost appears to be quite clean. Though the plaque size is somewhat larger than normal, the plaques are characteristic as in any control that has had its plasma removed (6318e).

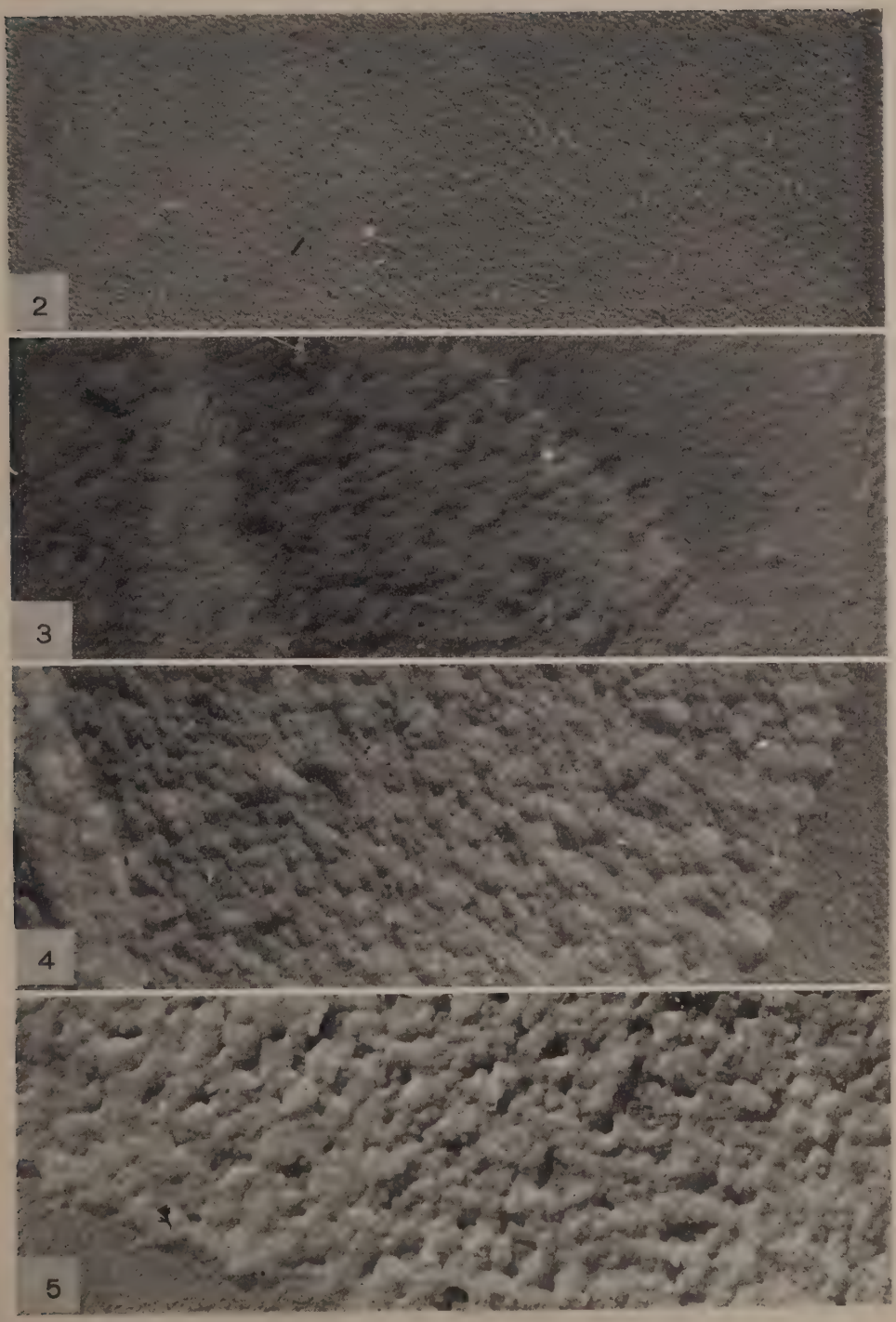
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PLATE 3

EXPLANATION OF FIGURES

The figures on this plate show the effect of brief exposure of the ghost to phosphotungstic acid. The action of phosphotungstic acid seems to be characterized by the plaques assuming a more discrete appearance. Also evident is a cleaner appearance of the entire surface and likewise the collodion (plate 2). What underlies this effect is not known. All of the figures on this plate are air dried and shadowed. Figures 7, 8, 9, all of which were of blood from different individuals, show the extremes in plaque size obtained, of all the bloods examined.

6 This is representative of the typical, general result with respect to plaque size. The plaques measure from 150 to 250 A in diameter. The large lighter regions are folds in the plasma membrane (6195c).

7 This indicates the minimum in plaque size obtained — ranging from 100–150 A in diameter (6266d).

8 These are plaques 250–350 A in diameter and are intermediate between the mean (fig. 6) and the large extreme (fig. 9) (6238c).

9 This shows the maximum plaque size observed. These vary from 300 to 450 A in diameter (6262b).

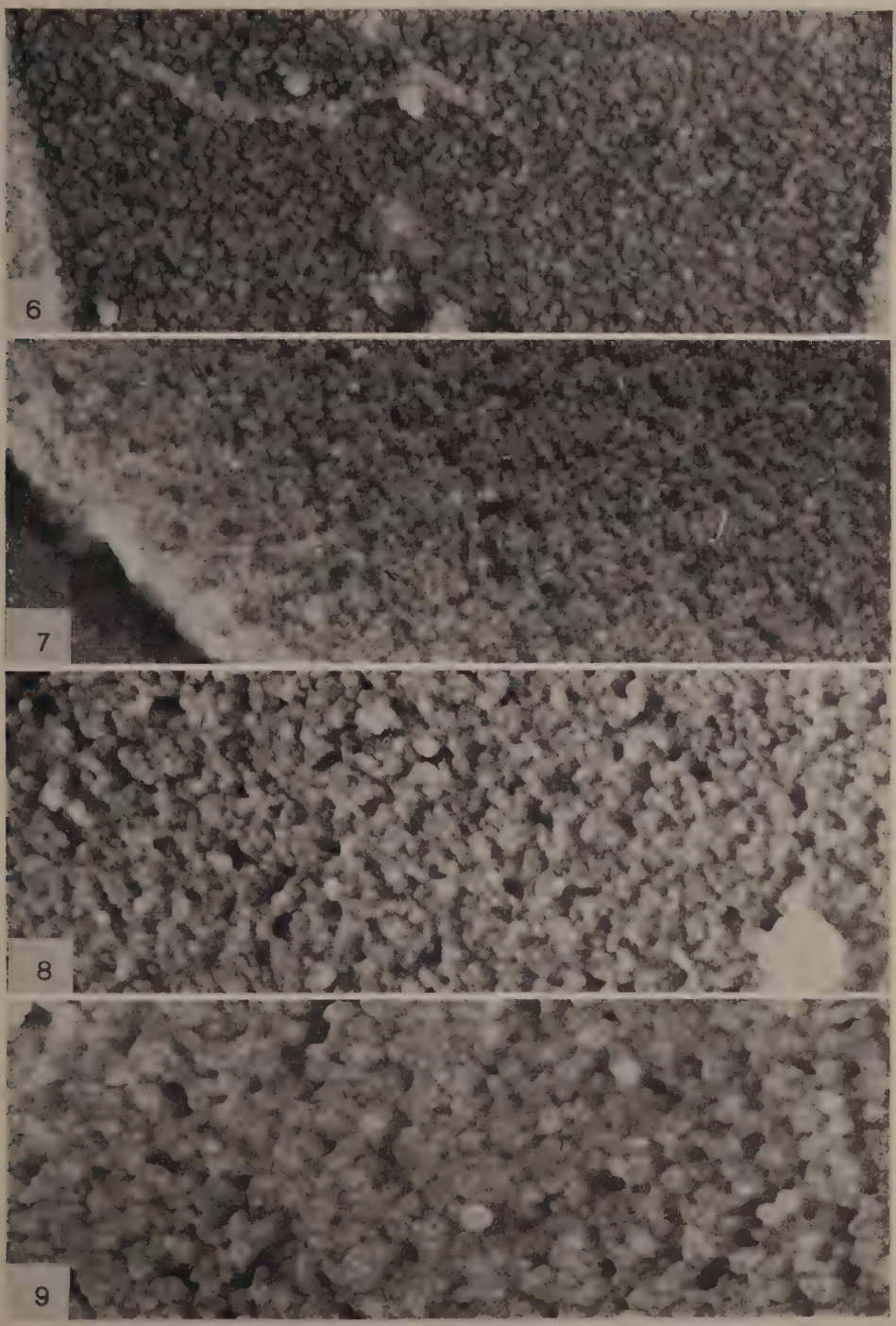
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PLATE 4

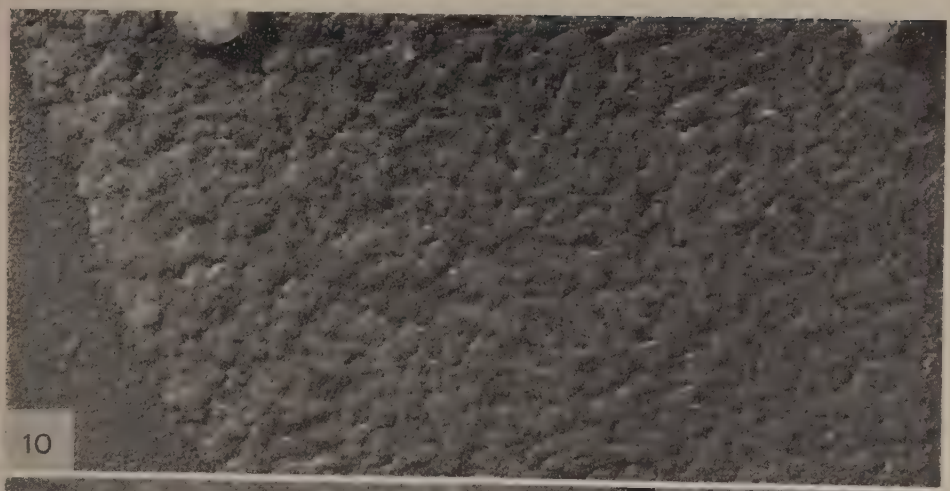
EXPLANATION OF FIGURES

These ghosts represent different treatments of the same sample of blood. This gives some idea of the uniformity of an individual's blood. These figures also show the correlation of the dense areas of the unshadowed (direct transmission) picture with the plaque regions in the shadowed specimen. The action of phosphotungstic acid is apparent. All are air dried.

10 Control shadowed (6132a).

11 Phosphotungstic acid. Unshadowed (6110b).

12 Phosphotungstic acid. Shadowed (6133a).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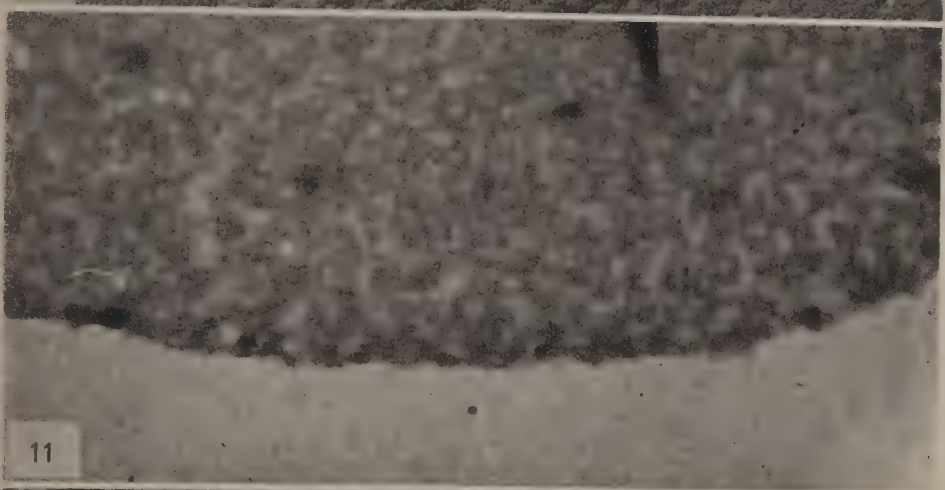
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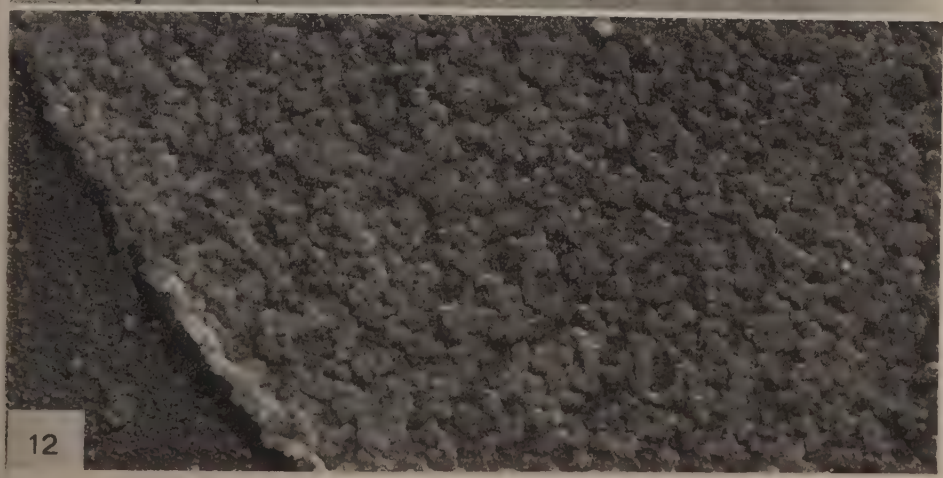
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PLATE 5

EXPLANATION OF FIGURES

13 Air dried. Shadowed. Treated with phosphotungstic acid (pH 2.8) in isotonic sodium chloride (6285b).

14 Air dried. Shadowed. Treated with phosphotungstic acid in isotonic sodium chloride at pH 7.4 (6291c).

15 Air dried. Shadowed. Treated with phosphotungstic acid at pH 7.4 (6284a).

It is evident that no readily observable changes have been introduced by isotonicity or control of the hydrogen ion concentration in the action of phosphotungstic acid. The cells shown are from the same sample of blood.

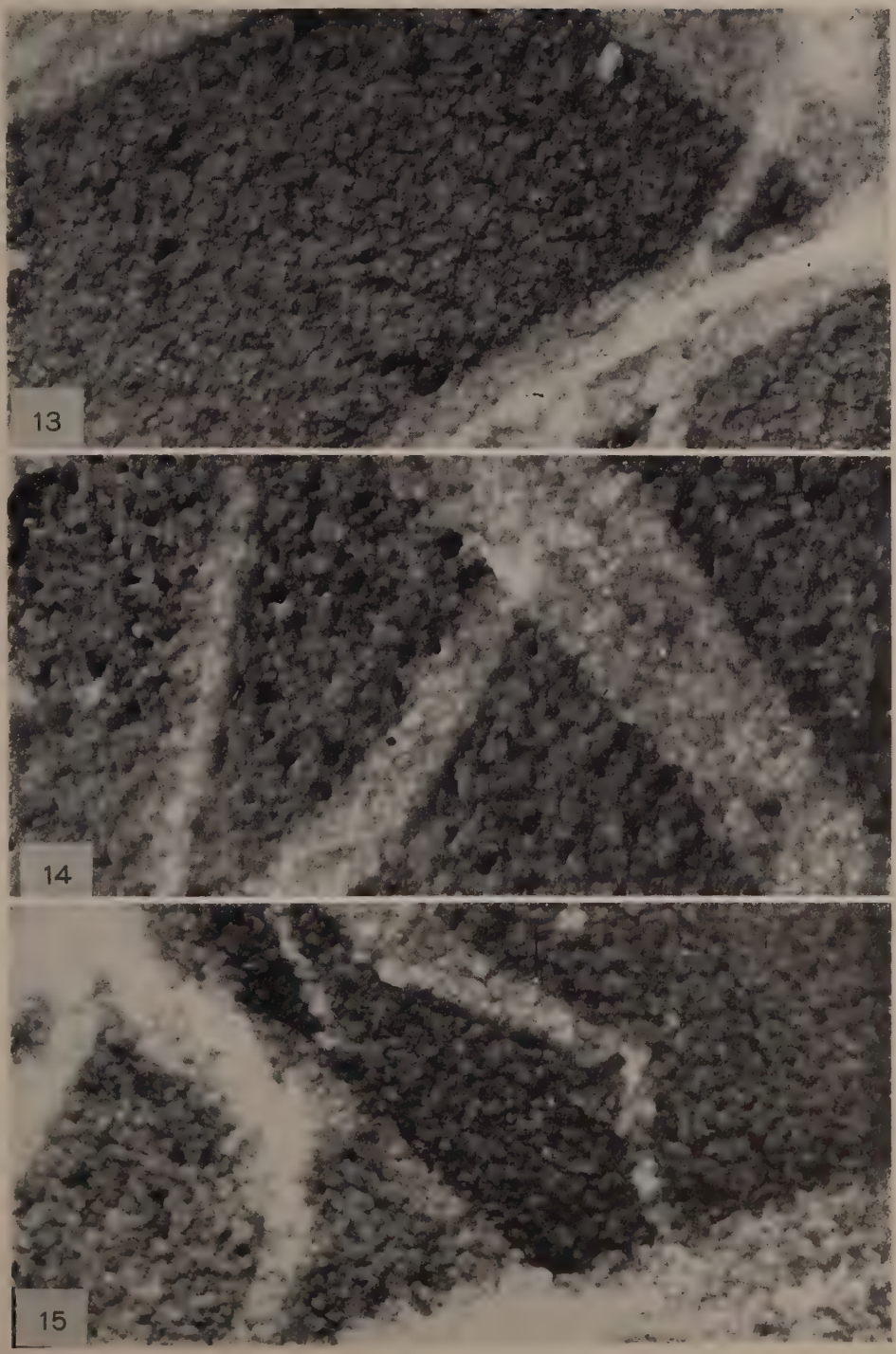
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PLATE 6

EXPLANATION OF FIGURES

16 Air dried. Shadowed. Exposed first to phosphotungstic acid, then washed and exposed to formalin (6265e).

17 Air dried. Shadowed. Exposed to phosphotungstic acid, then washed, and then to 70% alcohol (6263a).

18 Air dried. Shadowed. Exposed to 70% alcohol, washed, and then to PTA (6273e).

This emphasizes the importance attached to the order of treatments. Clearly, alcohol (which does not remove lipid from the ghost) introduces an artifact in the appearance of the surface. The pictures also demonstrate the rôle of phosphotungstic acid as a fixative.

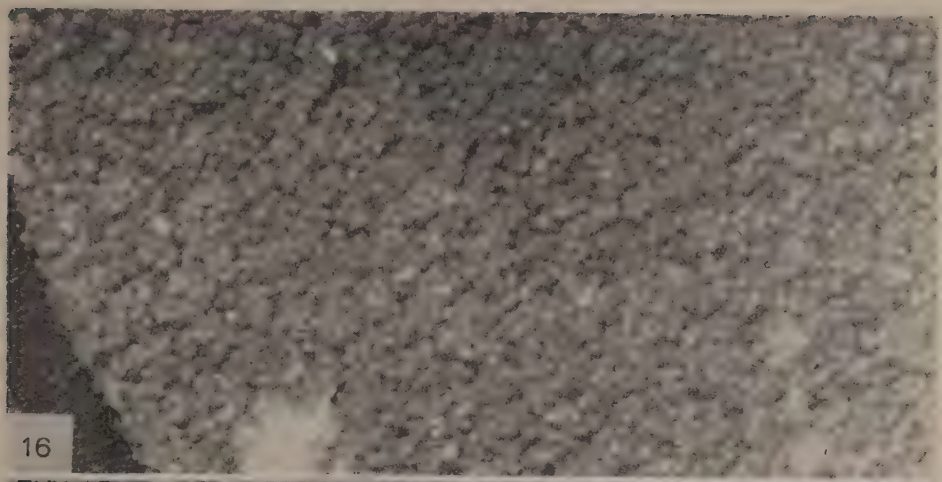
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PLATE 7

EXPLANATION OF FIGURES

19 Air dried. Shadowed. Treated in vapors of OsO_4. This shows that the action of OsO_4 is quite similar to that of PTA in the elaboration of plaque structure. This same result is obtained if the ghosts are immersed in phosphate buffer (pH 7.4) at the time of exposure to the vapors of OsO_4. The dense (white) cogwheel shaped particles are crystals of some, as yet, unidentified material (presumably a compound of osmium) which appeared on the underside of the collodion film (6054b).

20 Air dried. Phosphotungstic acid (pH 7.4). Unshadowed. Throughout the entire preparation of this ghost the pH was held constant at pH 7.4. Note that the ghost has a large coarse network rather than the characteristic close packing seen before. This is believed to be an artifact. It is not clear whether a rearrangement or loss of material is responsible for this result. This micrograph bears a strong resemblance to the results of Wolpers ('41) although the preparation of the specimens were not the same (6293e).

21 Air dried. Unshadowed. Exposed to phosphotungstic acid. Magnification 144,000 ×. Fibers are evident over the whole surface. They are of the order of 20 A in width (6306e).

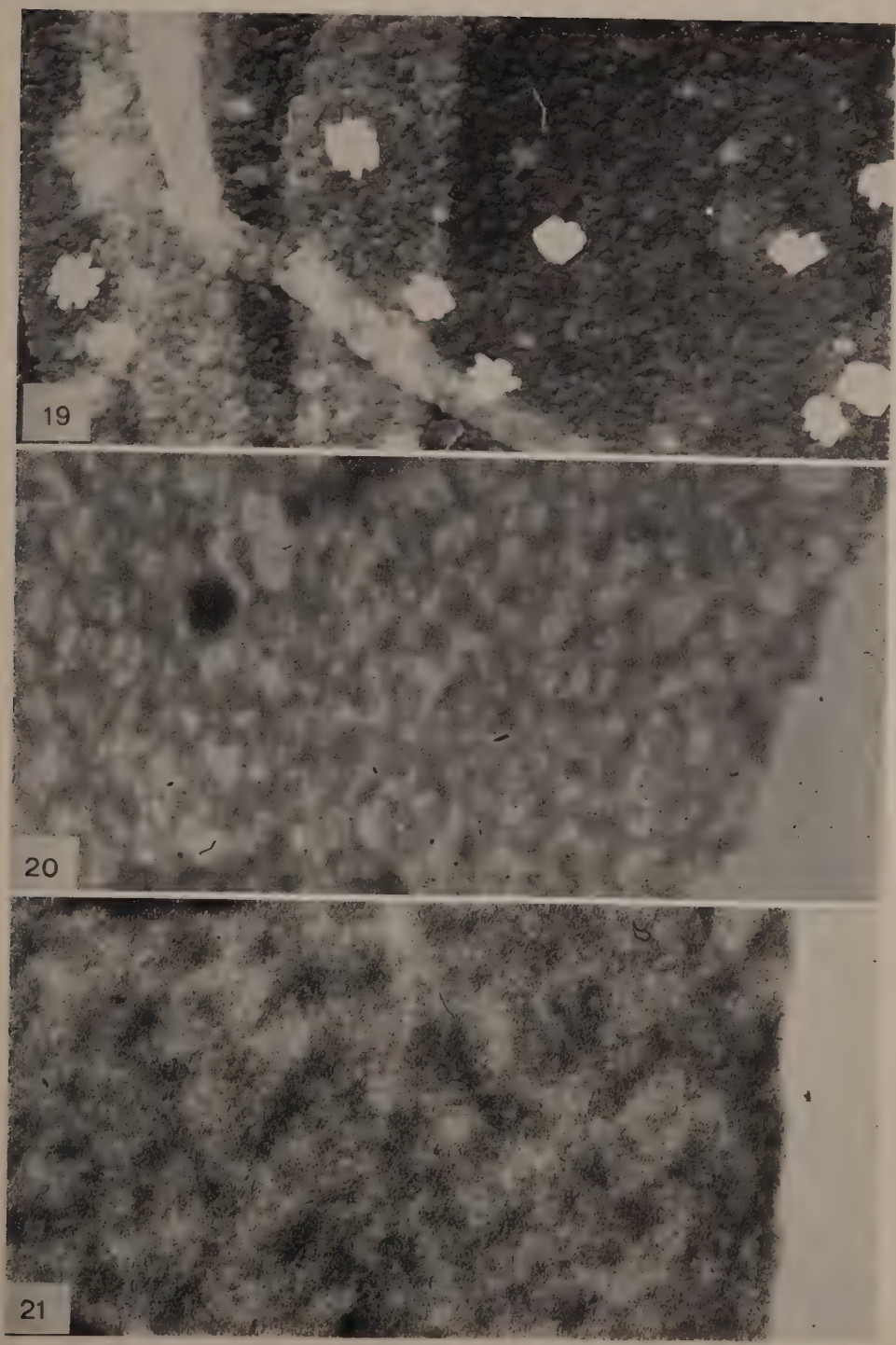
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PLATE 8

EXPLANATION OF FIGURES

22 and 23 Air dried. Unshadowed. Treated with phosphotungstic acid. These are two consecutive photographs of the same field, taken at 52,000 × on Kodalith emulsion. The magnification is 144,000 ×. Figure 23 is slightly under focus whereas figure 22 is very near focus. There are no shifts present in these micrographs. A comparison of the two pictures point for point shows that the fibers are present in both exposures and that the slight differences are consistent with the slight difference in focus. This means that the fibers are real structures at least insofar as they exist in the electron microscope specimen. If the electron optical magnification is varied maintaining the photographic grain size constant the fibers are found to change their dimension accordingly. This is further evidence for the reality of the fibers in the specimen (6305a, b).

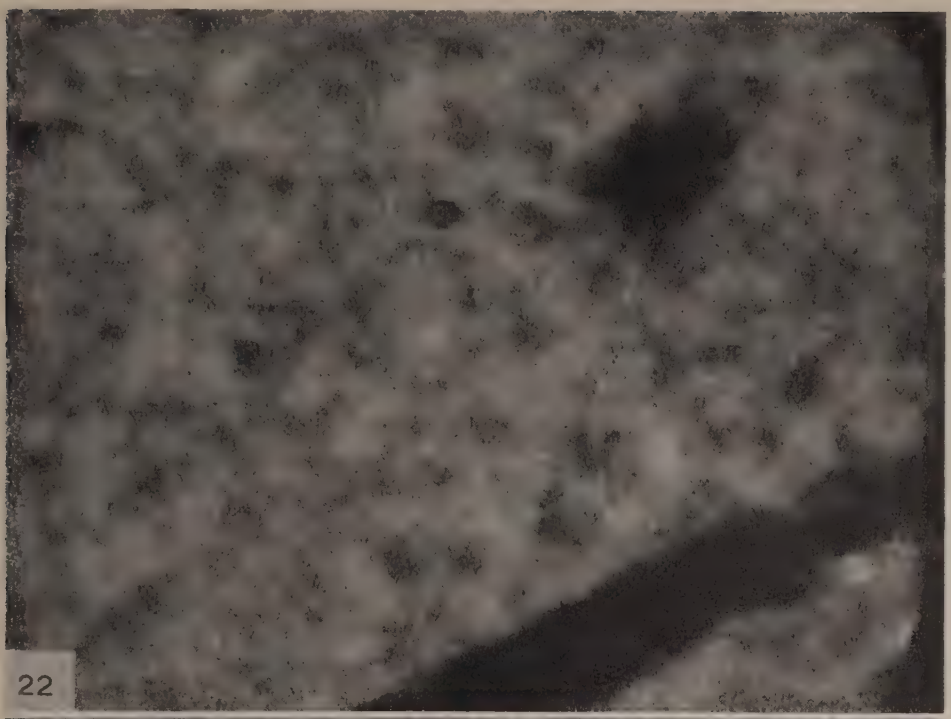
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PLATE 9

EXPLANATION OF FIGURES

24 Air dried. Shadowed. This ghost has been partially extracted with alcohol-ether-chloroform. Plaques can now be seen on the collodion membrane and a decreased population on the surface of the ghost (6147c).

25 Air dried. Shadowed. Extracted with alcohol-ether-chloroform *after* treatment with phosphotungstic acid. Presumably the same artifact introduced by PTA (viz. discreteness) is maintained. The plaques, as in figure 24, can be seen on the collodion and very few can be seen on the ghost proper. The plaques which are partially visible within the boundaries of the cell are those on the underside trapped between the remaining cell membrane and the collodion film. In the shadowed picture the fact that these have two membranes lying over them makes them less discrete (6264c).

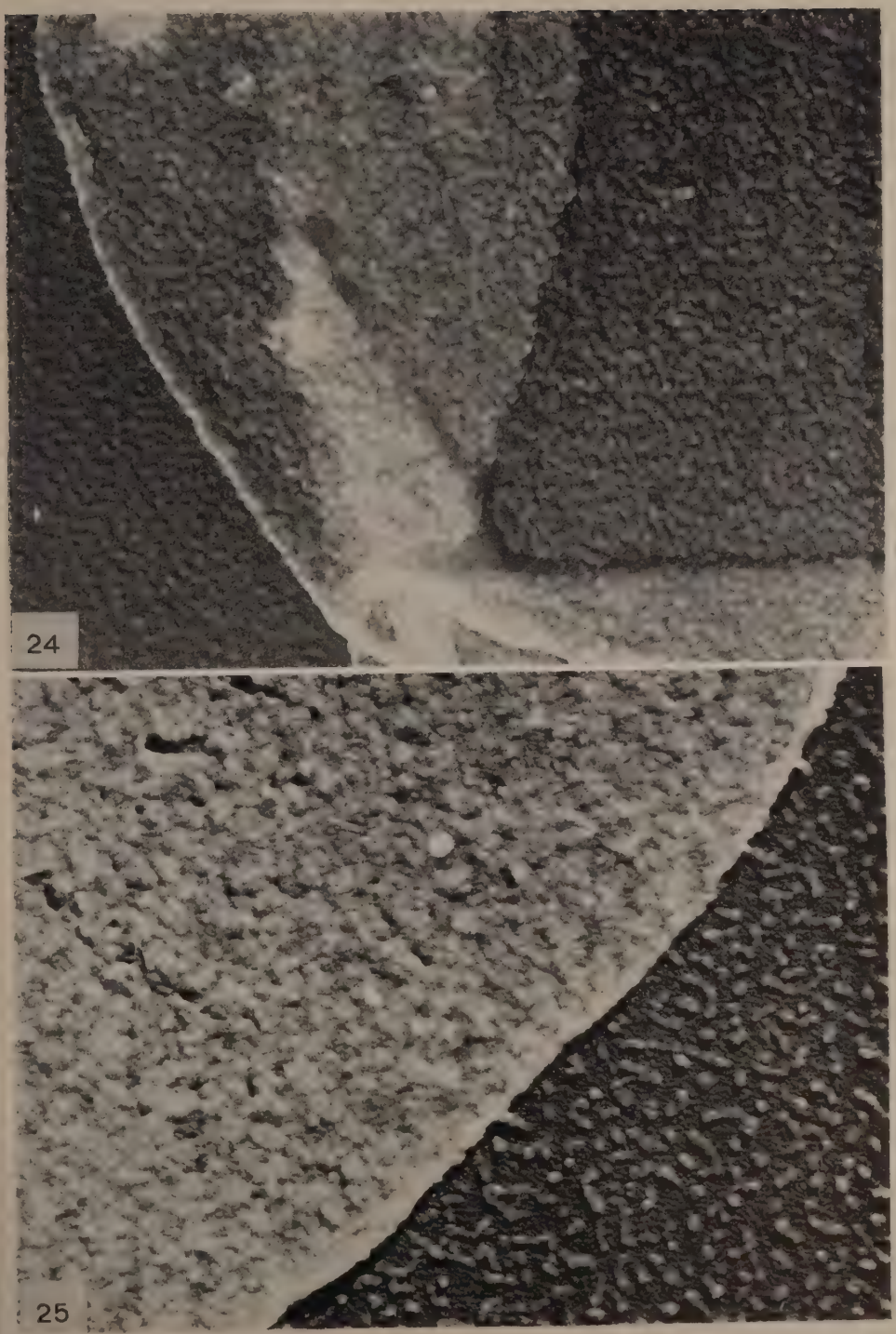
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PLATE 10

EXPLANATION OF FIGURES

26 Air dried. Shadowed. Partially extracted with alcohol-ether-chloroform *before* exposure to phosphotungstic acid. As before plaques have been removed from the ghost and can be seen on the collodion (6148d).

27 Enlargement of portion of figure 33 to 200,000 ×. The addition of PTA has elaborated some new structures not seen in figure 24. An organized network between and on plaques is now apparent. The dimension of the fibers composing the network is of the order of 25 A. The molecules responsible are yet to be identified.

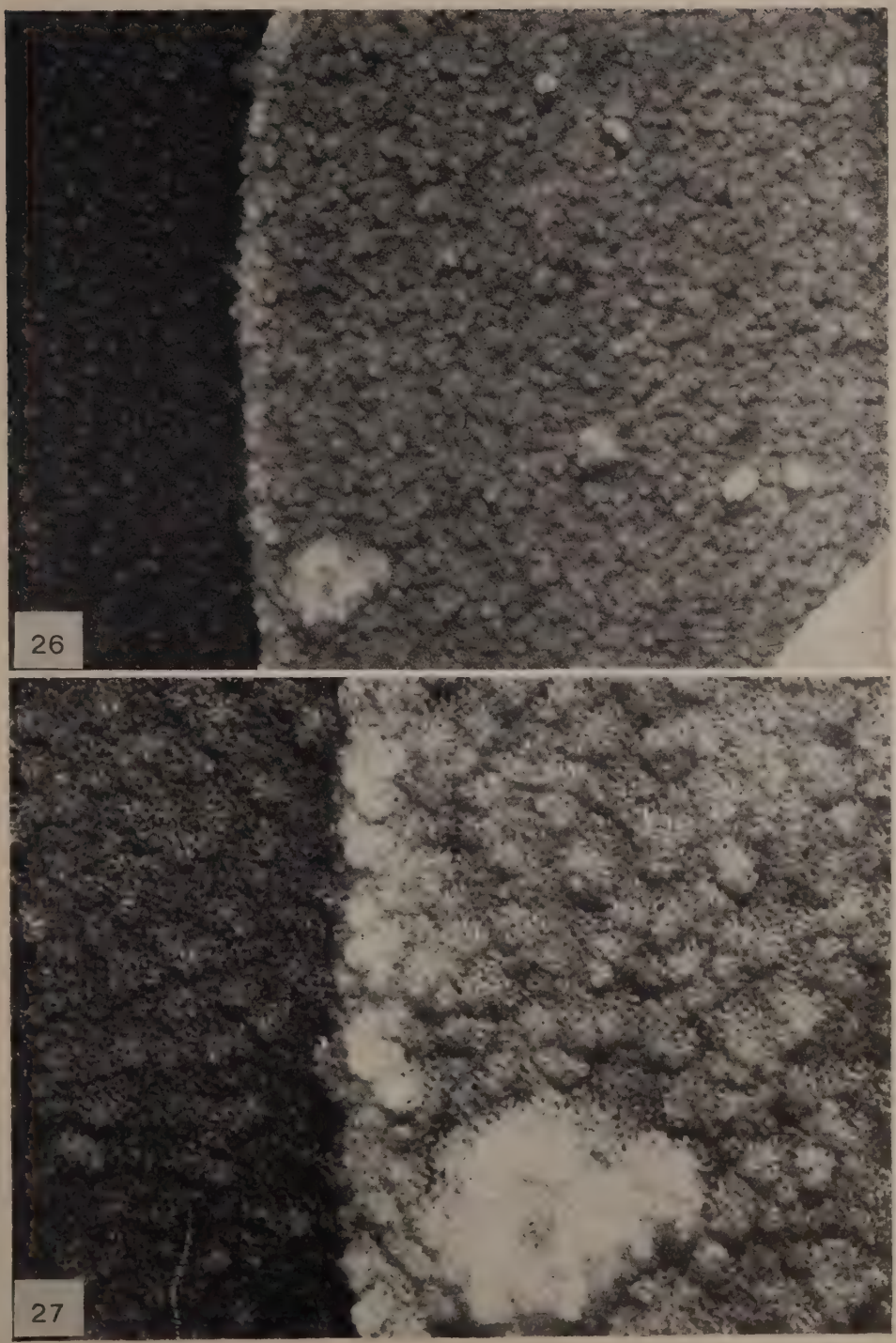
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PLATE 11

EXPLANATION OF FIGURES

28 Air dried. Shadowed. Treated with alcohol-chloroform. This picture shows that plaques are not removed by the action of alcohol-chloroform but ether is required for separation. If this micrograph is compared with a control this ghost appears somewhat cleaner (6144e).

29 Air dried. Shadowed. This ghost has been partially extracted and then exposed to formalin. Plaques can be observed on the collodion membrane. The surface of the ghost is altered and reminiscent of the action of alcohol alone (fig. 18). The significance of this picture is not yet understood (6278b).

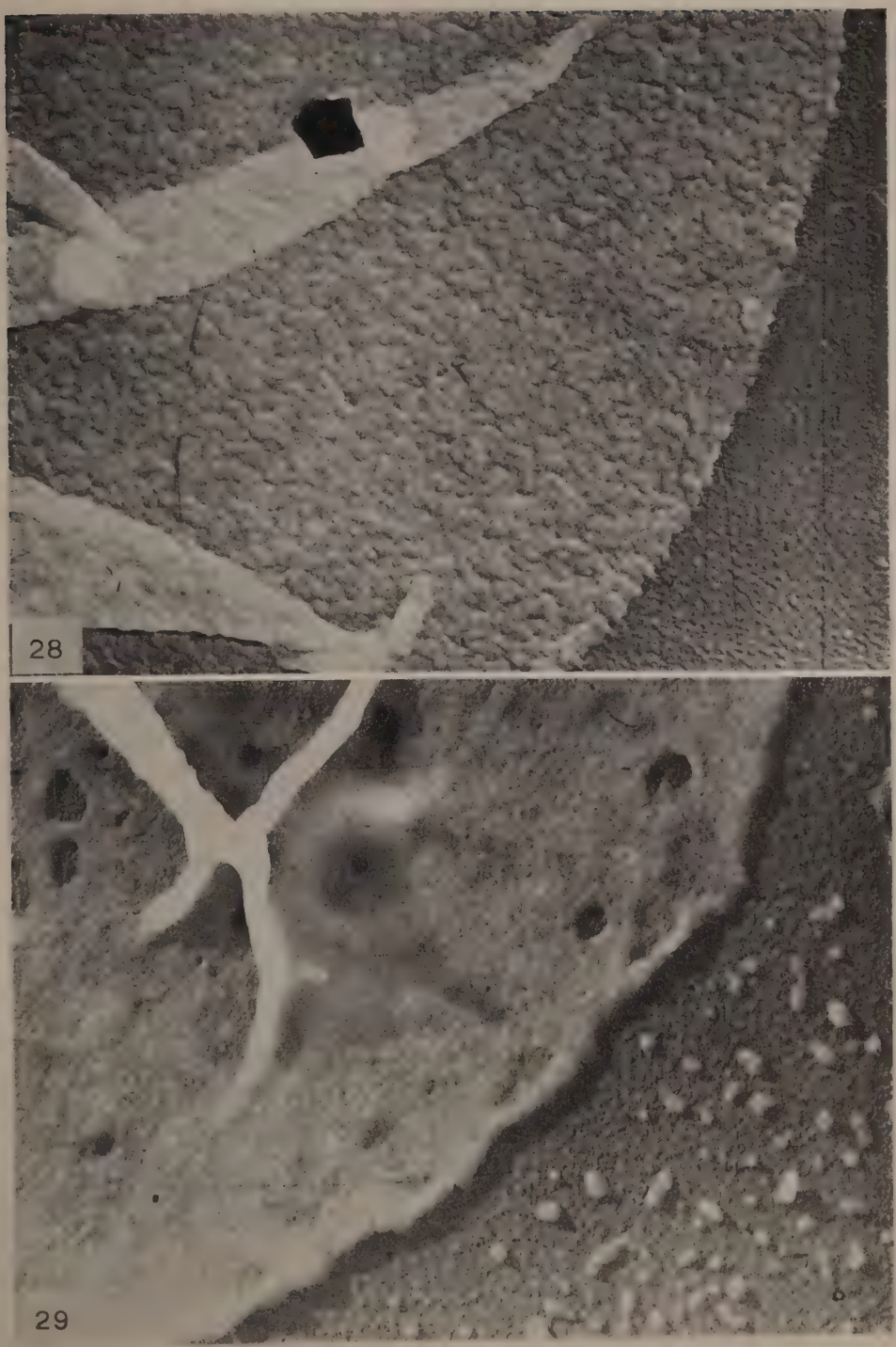
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PLATE 12

EXPLANATION OF FIGURES

30 Air dried. Shadowed. Rat cell treated with phosphotungstic acid (6234c).

31 Air dried. Shadowed. Groundhog cell treated with phosphotungstic acid (6233e).

These ghosts should be compared to human cells (plate 3). The rat appears qualitatively different. The groundhog has a mean plaque size larger than human. Rabbit ghosts (not shown) have a great similarity to human. In future studies these findings may suggest a molecular basis for species differences.

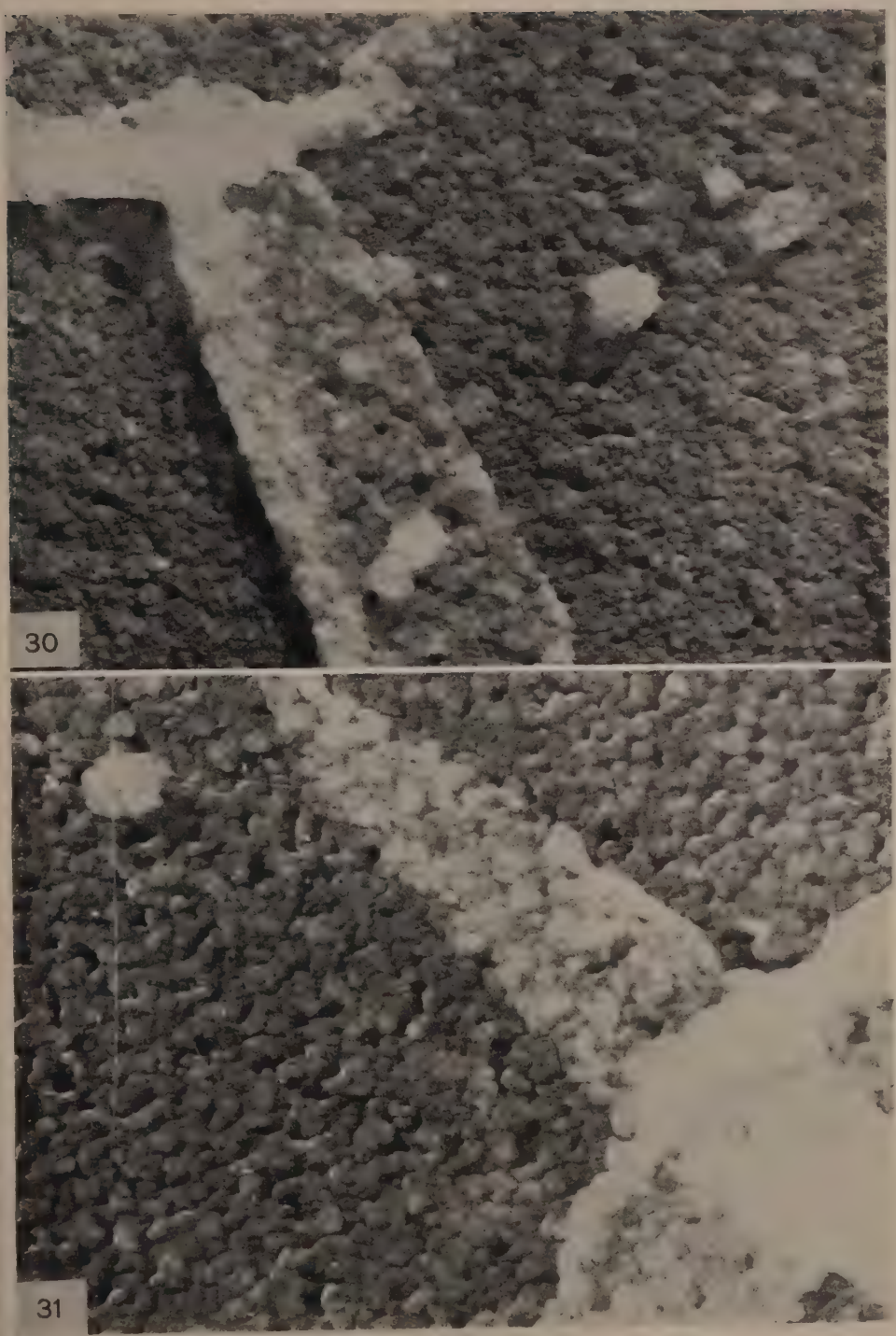
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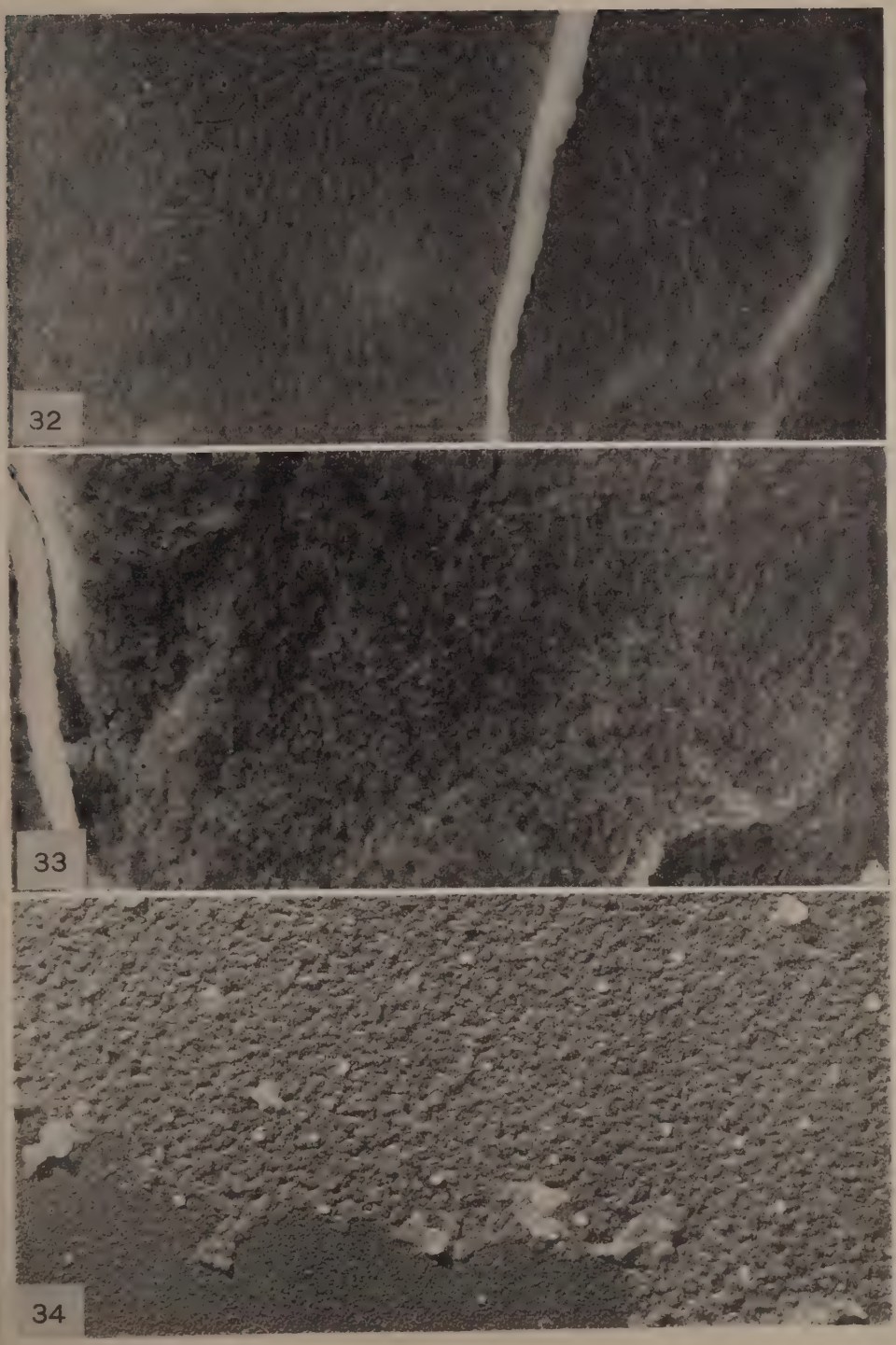
PLATE 13

EXPLANATION OF FIGURES

32 Frozen dried. Shadowed. Control. Like the air dried control this ghost shows the presence of plaques. The ghosts are spheres but crinkle under the electron beam (6166c).

33 Frozen dried. Shadowed. Treated with phosphotungstic acid. That phosphotungstic acid causes the plaques to become more discrete is apparent (plate 3) (6167b).

34 Frozen dried. Shadowed. This ghost maintained at neutrality was fixed in the vapors of OsO_4 before being frozen dried. Then the top was ripped off (by touching the ghost to glass) thus exposing the inner surface. This was then shadowed. The appearance of this surface leaves no doubt that the character of the inside is quite different from the outside. Plaques are nevertheless observable. Also present are osmophilic granules distributed over the surface (6324c).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THE ELECTRICAL ACTIVITY OF THE MUSCLE CELL MEMBRANE AT THE NEURO-MUSCULAR JUNCTION

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SIX FIGURES

INTRODUCTION

Previous experimental studies of neuro-muscular transmission indicate that valuable basic information may be derived from the analysis of membrane potential changes which occur during the transmission process. Important advances in this approach became possible when Kuffler succeeded in isolating an intact muscle fiber with its attached motor neuron. The results of the interesting work with this preparation have been summarized by Hunt and Kuffler ('50).

In Kuffler's experiments, the electrical changes occurring at the neuro-muscular junction during transmission were recorded by placing a small electrode on the surface of the muscle fiber at the end-plate. An important advantage of this technique is that a large number of records may be obtained from a single preparation. However, for accurate recording of the membrane potential an intracellular electrode is required.

By employing the technique described by Nastuk and Hodgkin ('50), membrane potential records have been obtained with an intracellular electrode placed at the neuro-muscular junction. The results of preliminary experiments have been given in earlier publications (Nastuk, '50a, b). At the same time Fatt and Katz ('50a, b) reported the results of similar experiments in which the above technique was used. More

recently these investigators have given a detailed and informative account of their work including an analysis of certain critical aspects of neuro-muscular transmission (Fatt and Katz, '51).

In the experiments where intracellular electrodes were employed, it was found that the action potentials which originate at the end-plate are smaller in magnitude than those which may be recorded from other regions of the muscle fiber. This contrasting behavior appears to have broad significance, therefore it is important to give assurance of the reliability of the recordings.

The data on membrane action potentials which are presented in this paper were obtained using high fidelity recording equipment. As a consequence, it becomes possible to describe with improved precision, the time course, rate of change, and magnitude of the membrane potential during neuro-muscular transmission, and during impulse propagation through a region of the muscle fiber distant from the end-plate. It will therefore be of interest to compare those data which are common to this report and to the report of Fatt and Katz ('51), and to discuss further the role of acetylcholine in neuro-muscular transmission.

METHODS

The method used was similar to that previously described (Nastuk and Hodgkin, '50). The sartorius muscle of the frog (Rana pipiens) with its attached motor nerve was dissected, and the muscle with its innermost side up was mounted in a dish containing Ringer's fluid of the following composition:

Na	116.55mM/L.
K	2.5
Ca	1.8
Cl	117.1
H_2PO_4	0.45
HPO_4	2.55

At 20-minute intervals during the course of the experiment the dish was drained and refilled with fresh solution. The

proximal attachment of the muscle was gripped in screw controlled forceps and the distal tendon was tied with a loop of thread which was held by a small clamp. The nerve, held in light forceps, was lifted from the fluid during the application of stimuli. A strip of silver foil which dipped into the Ringer solution served as the indifferent stimulating electrode. The entire assembly was mounted on a metal frame which was attached to the mechanical stage of a microscope. By this means it was possible to bring various sections of the muscle into the optical field.

It is necessary that the optical system have sufficient resolution if one is to see the termination of a neuron on a muscle cell. An ordinary stereoscopic microscope is inadequate in this respect. In this work acceptable visualization was obtained using a microscope with a 10 × objective (N.A. 0.25) and two 10 × oculars in a binocular arrangement. Adequate illumination was obtained from a 6 volt light source in combination with a condensing lens (N.A. 0.25).

The muscle cells were impaled at a 45° angle from the horizontal. This approach was necessary because of the short working distance between the microscope objective and the muscle and also because it permitted one to see the tip of the microelectrode clearly. Further, by this arrangement it is possible to avoid optical distortions produced at the point of entry of the microelectrode into the fluid since this troublesome area lies outside of the optical field.

A photograph of the apparatus is shown in figure 1. The probe which carries a microelectrode on its tip is mounted at a 45° angle in the micromanipulator. The coarse adjustments of the manipulator were used to maneuver the tip of the microelectrode close to the selected point on the surface of the muscle cell. The cell membrane was pierced using a fine screw adjustment to advance the probe along the 45° angle.

In a previous paper (Nastuk and Hodgkin, '50) it has been shown that the action potential is largely completed before appreciable muscle shortening occurs. However, during the

subsequent movement of the muscle there is considerable possibility of damaging the membrane of the impaled cell; in fact signs of such damage were often observed. To avoid the complications of injury it was a rule, in this study, to impale each cell only once.

The microelectrodes were of the same dimensions as those used in earlier work but some changes in the technique of

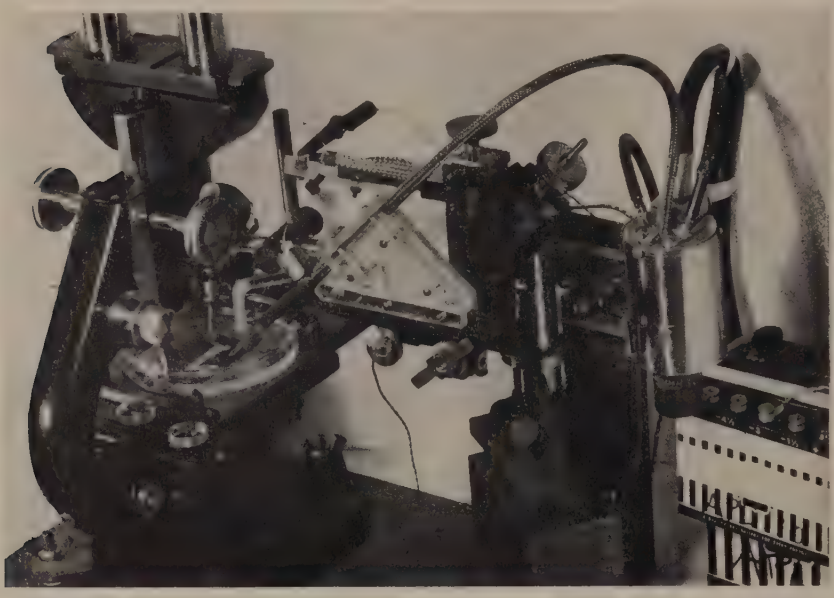

Fig. 1 Photograph of a portion of the apparatus showing the optical system, muscle chamber, arrangement of the electrodes, micromanipulator, and input cathode follower. The cathode follower shown is an earlier model which was later abandoned for an improved preamplifier. For further details see text.

manufacture were introduced. Originally the electrodes were filled by boiling them in 3 molar KCl. Such electrodes often show appreciable junction potentials when placed in contact with Ringer solution. Prolonged boiling seems to aggravate the condition to the point where junction potentials as high as 30–40 millivolts may be observed. In the filling process now used no heating is required and satisfactory electrodes are readily obtained.

The Pyrex tubing (O.D. 2 mm, I.D. 1.2 mm) from which the electrodes were drawn was cleaned by boiling and washing several times in distilled water. The tubing was then dried and the electrodes drawn using an electro-mechanical needle puller (Alexander and Nastuk, '53). The satisfactory electrodes were mounted on a holder and immersed in 3 molar KCl. Just before use the KCl solution was filtered through an alundum thimble to remove all debris. The shanks of the electrodes were filled by drawing off the air with a fine pipette, and after two hours' or more immersion the tips of the electrodes became sufficiently filled by capillary action. The small remaining gas bubble was dislodged by poking a very fine sharply pointed glass fiber down the shank of the microelectrode toward the tip. A few thrusts are usually sufficient to complete the filling process. Electrodes prepared by this method show lower junction potentials and greater stability than those prepared by the boiling procedure. The resistance of the electrodes commonly used varied between 6 and 15 megohms.

In the early experiments the probe was connected to the grid of a special cathode follower, the circuit of which has been described (Nastuk and Hodgkin, '50). With this type of cathode follower it becomes possible to reduce the input time constant to values in the vicinity of 70 μsecs. At first it was supposed that time constants of this order would be satisfactory. But analysis of the preliminary records showed that limitations in high frequency response introduced appreciable distortion in the recordings, and that faithful recording could only be accomplished with equipment having an overall time constant of less than 30 μsecs.

These recording requirements are more stringent than those given in an earlier paper (Nastuk and Hodgkin, '50). One reason for this difference is that the present experiments were carried out at 22–23°C. whereas in the above cited work the temperature range was 16–18°C. Another contributing factor is that the action potentials originating at the end-

plate differ in shape from those recorded at points distant from the end-plate.

With these considerations in mind, the input system was modified to include a compensation scheme by which means the overall time constant was consistently reduced to 15 μsecs. or less. In practice, the compensator was adjusted to the proper value before each recording was attempted, taking care to avoid overcompensation with the attendant possibility of overshoot in the system response. The grid current of the input cathode follower was maintained at less than 1×10^{-11} A. by adjustment of circuit constants and electrode voltages. Complete details of the input circuit have been given in another paper (Solms, Nastuk and Alexander, '53).

The action potential as recorded with an internal electrode includes the potential across the cell membrane and in addition, any potential in the extracellular fluid between the external surface of the membrane and the indifferent electrode. The magnitude of the "external action potential" was evaluated from control experiments in which the recording was done with the tip of the microelectrode placed at the outer surface of the muscle fiber. From 12 records obtained at points distant from the end-plate, the average external action potential was found to be 1 mV. A similar series of 12 records made at the end-plate gave an external action potential of 2 mV. To obtain the true maximum amplitude of the membrane potential it would therefore be necessary to add 1–2 mV. to the values measured with an internal electrode. However since the correction is small and uncertain no attempt was made to include it. The important point is that the correction is essentially the same whether action potentials are recorded at the end-plate or off it, and any difference in the magnitude of action potentials obtained from the two regions cannot be ascribed to differences in external action potentials.

The time base calibrator consisted of a 100 KC. crystal oscillator and suitable dividing multivibrators providing 1 msec. or 0.2 msec. marking pulses.

At the start and end of each experiment a calibration grid was obtained by applying 10 mV. steps between the indifferent electrode and ground and photographing a single stroke of the sweep at each potential setting. This was done for the x and y axes. An enlarged image of the grid was projected on a graph sheet and traced. The records were analyzed by projecting them on this grid.

RESULTS

On searching over the innermost surface of the muscle one may find emerging from a nerve bundle, a single neuron which extends a short distance and appears to terminate on a muscle cell. This is shown in the photomicrograph of figure 2. At the optical magnification used, and in the absence of stain, one does not see the fine terminal branches of the neuron. In this work it was assumed that the center of the end-plate corresponded to the point at which the neuron appeared to terminate. The validity of this assumption was substantiated by rough mapping of the end-plate zones of a number of cells. This was done by applying minute amounts of acetylcholine to the surface of the cell in and around the end-plate zone end noting the presence or absence of twitching. Details of the technique have been given in another report (Nastuk, '51).

The record shown in figure 3 is representative of the results obtained when the resting and action potentials of the muscle cell are measured with the internal microelectrode placed at the center of the end-plate. In this record the membrane action potential was produced by stimulating the motor nerve.

Considering the action potential it will be seen that the first sign of electrical activity involves a rather sudden depolarization of the muscle cell membrane. This primary depolarization has been termed the end-plate potential. As the depolarization proceeds, a critical membrane potential is reached following which the rate of depolarization increases. Further, it can be seen that the action potential is of such magnitude that the sign of the membrane potential is reversed

for a brief period, i.e., overshoot occurs. Beyond this point the repolarization of the membrane is not a smooth process; near zero membrane potential the repolarization is slowed giving the appearance of a hump in the curve. A plot of another typical action potential containing the nomenclature

Fig. 2 Photomicrograph of a portion of a sartorius muscle showing a nerve bundle from which a single neuron emerges and runs a short distance before terminating at the end-plate. To the left is a microelectrode with its tip pointed at the center of the end-plate. The microelectrode appears as a conical shadow because practically all of it lies outside of the focal plane.

used in this paper to describe certain aspects of the recordings is given in figure 4.

Average data from records of resting and action potentials obtained at the end-plate and from points distant from the end-plate are presented in table 1. Certain aspects of these

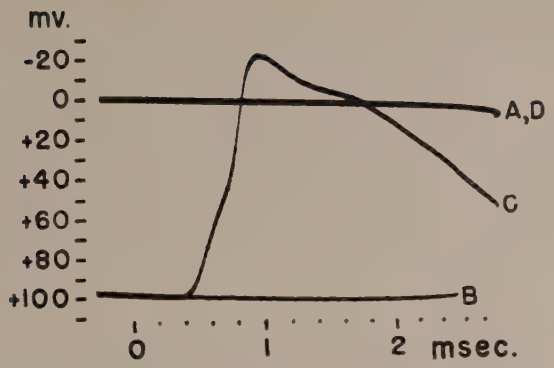

Fig. 3 A typical resting and action potential record obtained at the center of the end-plate. A, D are records with the microelectrode outside the fiber at the beginning and end of the experiment. B and C are records with the microelectrode inside the fiber. In B the fiber is at rest. C shows the activity which results from stimulation of the motor nerve. The potentials are given as outside potential minus inside potential.

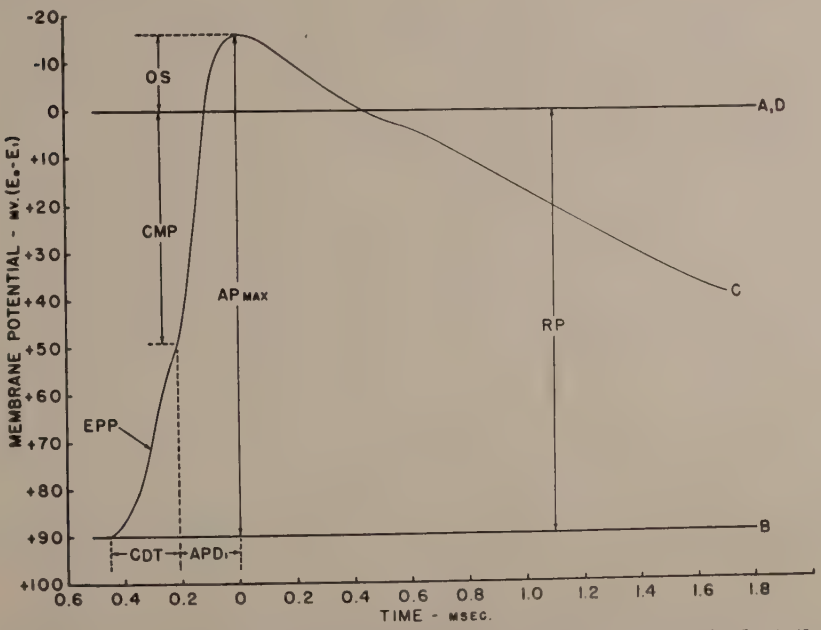

Fig. 4 Plot of a typical resting and action potential record obtained at the center of the end-plate which illustrates the nomenclature used in this paper.

A, B, C, D, as in figure 3.
APD_1, action potential duration — rising phase (elapsed time between the dashed vertical lines).
APmax., maximum amplitude of the action potential.
CDT, critical depolarization time (elapsed time between the dashed vertical lines).
CMP, critical membrane potential (read at the point of inflection).
EPP, end-plate potential.
OS, overshoot.
RP, resting potential.

Zero time was arbitrarily set at the crest of the action potential to facilitate the comparison of different records.

data require further comment. Each muscle provided several fibers from which records were obtained at end-plates. The same muscle also provided an ample number of additional fibers from which recordings were obtained at sites distant from the end-plate. The data from end-plates were divided into two groups as indicated, and all data representing results obtained distant from the end-plate were placed in the third group. The above procedure was followed in order to control possible variations in the muscle preparations and in the experimental conditions.

It may be seen that the resting potential at the end-plate is practically identical with that of other regions of the cell. The small difference is not statistically significant. On the other hand, an interesting difference in membrane behavior is revealed by the fact that action potentials which originate at the end-plate are smaller in magnitude than those observed at points distant from this zone.

During the first phase of membrane activity known as the end-plate potential, the membrane is depolarized to a critical value of + 47 mV. (C.M.P.). The critical membrane potential is read at the point of inflection as indicated in figure 4. Although this point was obtained by inspection of projected enlargements of the records, the average value obtained in a duplicate determination done at a later date deviated by less than a millivolt from the original figure.

The membrane potential is reduced from the resting to the critical level in 0.29 msec. This interval has been termed critical depolarization time (C.D.T.). A duplicate determination of the average value of C.D.T. deviated from the original figure by less than 0.01 msec.

The maximum rates of rise of the end-plate potential and the propagated action potential given in table 1 were obtained by graphical analysis. In every case the maximum rate of rise of the end-plate potential was less than that of the initiated action potential. Also, in practically all of the cases the end-plate potential achieves its maximum rate of rise before the membrane potential reaches the critical level.

TABLE 1

Average characteristics of membrane potentials recorded at the end-plate and distant from the end-plate

	CENTER OF END-PLATE	N	35μ FROM CENTER OF END-PLATE	N	DISTANT FROM END-PLATE	N
Resting potential (RP) — mV.	+ 94.6 ± 0.8 *	29	+ 94.9 ± 0.9	29	+ 92.8 ± 0.5	60
Action potential (APmax) — mV.	120.9 ± 1.5	30	117.4 ± 1.4	29	130.8 ± 0.5	56
Potential difference across the active membrane at crest of spike (OS) — mV.	— 25.9 ± 1.3	29	— 22.7 ± 1.7	29	— 37.9 ± 0.7	56
Critical depolarization time (CDT) — msec.	0.30 ± 0.008	28	0.28 ± 0.01	27		
Critical membrane potential (CMP) — mV.	+ 46 ± 1	28	+ 48 ± 1.4	27		
Duration of the rising phase of the action potential (APD_1) — msec.	0.23 ± 0.004	29	0.18 ± 0.006	27		
Maximum rate of rise of end-plate potential — V./sec.	220 ± 9	29	250 ± 12	27		
Membrane potential at which rate of rise of end-plate potential is a maximum — mV.	+ 71 ± 1	28	+ 70 ± 1.5	27		
Maximum rate of rise of action potential — V./sec.	670 ± 22	29	750 ± 31	29	650 ± 25	52
Membrane potential at which rate of rise of action potential is a maximum — mV.	+10 ± 1.2	28	+ 12 ± 2.0	29	+ 8 ± 1.0	52
Time taken for spike to rise from 20% to 100% of crest — msec.					0.33 ± 0.006	52
Temperature — °C. (average)	22.3		22.9		22.6	

N = number of observations.

* = standard error of mean.

Potential difference across the membrane is given as outside potential minus inside potential.

In a few records the slope of the end-plate potential kept increasing until the critical membrane potential was reached at which point the rate of change of membrane potential rose sharply.

On comparing the records obtained from individual end-plates of the same muscle, it is found that there is considerable variation in the maximum rate of depolarization achieved during the rise of the end-plate potential and consequently there is also variation in the critical depolarization time. It is reasonable to suppose that such variations represent differences in the intensity of the transmission process at individual end-plates. On the other hand these variations might conceivably be explained on the basis that the zone of most intense transmitter activity because of its small size is difficult to locate, and that as one moves away from this zone, the transmitter activity diminishes rather rapidly.

To aid in deciding which of the above possibilities was most nearly correct, a group of recordings was taken with the microelectrode placed 35 μ from the center of the end-plate. The results obtained (table 1) are in good agreement with those obtained at the center of the end-plate. One may conclude, therefore, that over a distance of at least 70 μ the transmitter intensity at an individual end-plate is substantially uniform, but that among a group of end-plates the transmitter intensity varies considerably.

A rather interesting result shown in table 1 has to do with the maximum rate of rise of the action potential recorded at the end-plate and distant from it. Assuming that the data obtained at the center of the end-plate and at 35 μ from the center may be grouped, the average maximum rate of rise is 710 ± 19 V./sec. This value may be compared with 650 ± 25 V./sec. for action potentials recorded distant from the end-plate. Statistical analysis shows that the difference between these sets of data is of doubtful significance.

The average rate of rise of action potentials recorded at 35 μ from the center of the end-plate is 750 ± 31 V./sec. (table 1). Similar data from 25 fibers of the same muscles

but taken distant from the end-plate gave 670 ± 12 V./sec. In this case statistical analysis shows that the difference between these sets of data is significant.

Under the circumstances it seems only safe to say that the maximum rate of rise of action potentials originating at the end-plate is perhaps slightly greater compared with the action potentials traversing other regions of the cell. In any case, the important point to be noted is that the difference is small. To demonstrate it conclusively, a larger number of better controlled experiments, and more precise analytical technique are required.

A comparison of the action potentials recorded at the end-plate with those recorded at a point distant from the end-plate shows that in the former case the average rate of repolarization is less. In fact, membrane repolarization at the end-plate is not only slowed but the curve has a peculiar form. These differences are brought out by the plot shown in figure 5. To prepare this plot the time base of each recording was divided into 0.1 msec. intervals, starting from the apex of the spike, and the membrane potential was read at each interval. Each point on the plot of figure 5 represents an average membrane potential obtained from several recordings. Although this type of averaging results in some loss of the detail which would be shown in any individual record, it nevertheless clearly brings out the difference in the time course of membrane repolarization at and away from the end-plate.

It has been pointed out in discussing the sample record shown in figure 3 that the end-plate potential begins as a rather abrupt depolarization of the muscle cell membrane. This observation is of special interest when certain anatomical factors are considered. Couteaux ('47) has shown that the motor neuron and its finely divided terminal branches lie in parallel relation with the muscle cell. One might expect, therefore, that as a nerve impulse is transmitted along the neuronal termination, the accompanying eddy currents would produce initially, an increase in the muscle cell membrane

potential. Careful examination of the action potential records reveals no evidence of such passive polarization. This is well illustrated in a typical action potential obtained at increased amplification (fig. 6). With such recording one may easily

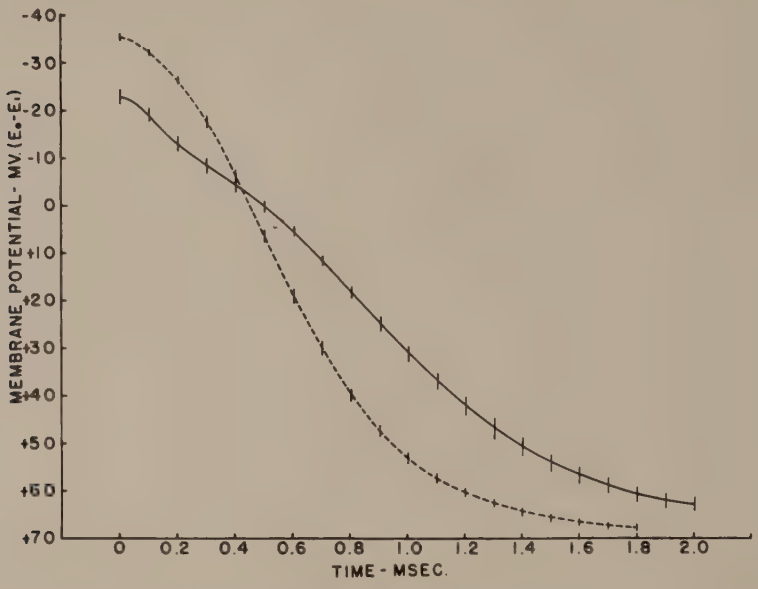

Fig. 5 Plot showing the falling phase of action potentials neurally initiated at the end-plate (solid line), and action potentials propagated through zones distant from the end-plate (dashed line). The origin of the time scale corresponds with the crest of the action potential. For the solid line each point represents the mean of 17 records, points on the dashed line represent the mean of 15 records. The length of the vertical bars is twice the standard error of the mean.

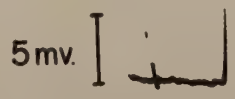

Fig. 6 Record of the foot of the end-plate potential obtained at high amplification. The stimulus artifact appears shortly after the beginning of the trace.

demonstrate membrane potential changes of 1 mV. and possibly 0.2 mV. It appears then that the eddy currents which accompany the nerve impulse do not exert an important influence on the membrane potential of the muscle cell.

DISCUSSION

The terminal event in neuro-muscular transmission is the production of a membrane action potential which is propagated along the muscle fiber. To bring this about, the excitability of the muscle cell membrane must be raised to the threshold value or putting it differently, the membrane potential must be reduced to a critical level. The view generally held at present is that subsequent to the arrival of a nerve impulse at the neural terminations a small quantity of acetylcholine is released which reacts quickly with the muscle cell membrane at the end-plate. As a result a primary reduction in membrane potential occurs (end-plate potential), and if the membrane is sufficiently depolarized, a propagated action potential is initiated.

It is well known that topical application of acetylcholine to the end-plate area causes membrane depolarization. Recently this has been demonstrated with single fibers using an internal electrode to record the membrane potential (Nastuk, '51). A reduction in membrane potential requires a transfer, across the membrane, of positive charge moving inward, or negative charge moving outward or both in combination. There then arises the question of the mechanism by which acetylcholine can give rise to the transfer of electric charge across the membrane. The data on the time course of membrane potential changes at the neuro-muscular junction provide some important facts, the interpretation of which has bearing on this problem.

The data in the first column of table 1 indicate that the membrane in the vicinity of the end-plate is in a critical state when the potential across it is + 46 mV. (outside potential — inside potential). In the most nearly comparable experiments reported by Fatt and Katz ('51), the critical membrane potential was found to be + 58 mV. The difference between these values may possibly be explained on the basis that the Ringer solution used by Fatt and Katz is not identical with that used in this laboratory. However, part of the discrepancy results from the fact that the records obtained by Fatt

and Katz were to some extent distorted by their recording equipment. The magnitude of this error has been pointed out by them.

It is interesting to compare the critical membrane potential given in this paper with certain other experimental results presented by Fatt and Katz ('51). They showed that the application of a cathodal current to the muscle fiber results in the production of a propagated action potential if the membrane potential is reduced to + 49 mV. This figure agrees closely with the value of + 46 mV. reported in table 1. From these data it would appear that the muscle fiber membrane along its entire length has a characteristic critical membrane potential in the range of + 46 to + 49 mV. when placed under the condition of these experiments.

Utilizing the above conclusion one may suppose that the following sequence of events occurs at the end-plate. An area of membrane lying within the end-plate zone reacts with released acetylcholine and undergoes active depolarization due to, say, a penetration of sodium ions. An adjacent area of membrane, part of which may lie within the end-plate patch, is passively depolarized since it is electrically coupled to the actively depolarized patch. During this phase the rate of change of membrane potential as measured at the center of the end-plate reflects the combined influences of penetrating positive charge and the passive discharge of the surrounding membrane. As the surrounding membrane continues to be passively discharged its potential finally reaches a critical level of about + 47 mV. At this point the depolarization in the surrounds changes from a passive to an active process involving the entry of sodium ions. Consequently there is an increase in the rate of change of membrane potential at the end-plate since both the membrane area which is modified by acetylcholine and the surrounding membrane now come into cooperation in initiating a propagated action potential.

Assuming that acetylcholine increases the permeability of the end-plate membrane for sodium ions, a high rate of change

of membrane potential would be expected at the time when this area and the surrounding membrane are cooperating in initiating a propagated action potential. Thus the maximum rate of depolarization would be greater for action potentials arising at the end-plate compared with those initiated off the end-plate by electrical stimulation. The data of Fatt and Katz ('51) indicate that for locally initiated action potentials the rate of rise does not differ from that achieved in conducted action potentials. It seems permissible, therefore, to compare the maximum rate of rise achieved at the end-plate following nerve stimulation, with that achieved during the conduction of an action potential through a zone distant from the end-plate.

The data in table 1 show that the maximum rate of change of the membrane potential is 670 V./sec. at the center of the end-plate, and 750 V./sec. 35 μ from the center. The average of these results gives 710 V./sec. which is 9% greater than the 650 V./sec. rate of rise for action potentials off the end-plate. It must be pointed out that statistical analysis indicates that the difference between these means is of doubtful significance. In any event, it may be emphasized that the difference is not great.

A possible explanation for this result is that during the rise of an action potential at the end-plate, only a small part of the total membrane area engaged in active sodium transport has reacted with acetylcholine. In this area of membrane the sodium permeability might be appreciably greater than that of the surrounds but its small size limits its effect on the overall sodium permeability. A more likely explanation is that a larger fraction of the active membrane has reacted with acetylcholine but that in this area the sodium permeability is only moderately increased with respect to the unreacted portion.

The effect of acetylcholine on membrane behavior is also brought out by the fact that action potentials elicited at the end-plate via nerve stimulation are smaller in magnitude than those which are propagated through the remainder of the

muscle fiber. Off the end-plate the active membrane potential reaches a maximum of —38 mV., on the end-plate the maximum is only —24 mV., a reduction of 14 mV. It may be noted that the values reported in table 1 for the resting potential and maximum active membrane potential both at the end-plate and off it, are significantly greater than those given by Fatt and Katz ('51, table 9). However, as regards the *reduction* in active membrane potential at the end-plate, the results reported in this paper are in excellent agreement with the value of 13 mV. found by Fatt and Katz.

In seeking an explanation for the reduction in active membrane potential at the end-plate several possibilities must be considered. As Fatt and Katz ('51) have pointed out, it might be supposed that the patch of end-plate membrane not only acts as a recipient for acetylcholine, but has additional characteristics which make its behavior quantitatively different from that of the remainder of the muscle fiber membrane. However, this idea was not supported by their experimental test of it.

Similar experiments have been conducted in this laboratory. The membrane potential was measured at the center of the end-plate to obtain the response during the transit of an action potential which was initiated by applying an electrical stimulus to the muscle fiber at a point 1 mm from the center of the end-plate. As regards magnitude, and maximum rate of rise and fall, this group of action potentials was not significantly different from a second group recorded in the same manner but at an end-plate free region of the muscle fiber. These results give no indication that the end-plate membrane has distinctive properties. However, the question cannot be completely settled for it is conceivable that a specialized end-plate membrane could exist but because of its small area, its influence is not large enough to be detected in the above experiments. What these results do indicate is that the propagation of an action potential through the end-plate zone does not trigger the neural mechanism which releases acetylcholine. If acetylcholine were released, these action poten-

tials would be expected to have different form and reduced amplitude.

Fatt and Katz ('51) considered another explanation for the reduction in the active membrane potential at the end-plate; an action potential is small at its point of origin and it increases in size after being conducted a short distance. Their experimental records obtained 50 μ from the point of application of a stimulus provide no evidence of such behavior.

From the preceding argument one is drawn to the conclusion that the reduction in active membrane potential shown at the end-plate reflects an alteration in membrane behavior produced by its reaction with acetylcholine. Fatt and Katz ('51) have postulated that the end-plate membrane, although insensitive to applied electric current, is nonetheless capable of reacting with acetylcholine. Following such reaction, its permeability is greatly increased to all species of ions both positively and negatively charged. In their view it is this "short circuiting" effect which accounts for the reduction of the active membrane potential.

A salient feature of the hypothesis proposed by Fatt and Katz is that acetylcholine exerts its depolarizing action by causing a simple membrane breakdown. Support for this idea was derived from the experiments of Fatt ('50). Using a whole muscle preparation bathed in Ringer solution in which the sodium chloride was replaced by an osmotically equivalent amount of glucose, he found that some reduction of the end-plate membrane potential still occurred when acetylcholine was applied. The reduction was about half that which could be produced in control experiments in which normal Ringer solution was used. From this Fatt concluded that sodium ions reinforce the depolarization produced by acetylcholine but are not necessary for its action.

The relation between external sodium ions and the depolarizing action of acetylcholine has also been tested in this laboratory using a different technique (Nastuk, '51). In these experiments acetylcholine was applied to a single end-plate

while simultaneously recording the membrane potential with an internal microelectrode located 200 μ from the center of the junction. With the muscle in normal Ringer solution a quantity of acetylcholine was applied to the end-plate which was sufficient to reduce the membrane potential by 30 mV. At this level of depolarization no propagated action potentials were initiated. Using a Ringer solution in which the sodium chloride was replaced by sucrose, application of the same quantity of acetylcholine reduced the membrane potential by only 2–3 mV. If the amount of applied acetylcholine was increased 5-fold, the drop in membrane potential was 10 mV., but in this case the change was not immediately reversible since the membrane potential increased only slightly when the acetylcholine was removed.

The small change in membrane potential produced by acetylcholine in the absence of external sodium can be explained by assuming that the end-plate membrane is highly permeable to acetylcholine ions. The membrane potential was reduced 2–3 mV. by continuous application of acetylcholine to the fiber surface at a rate roughly calculated to be 2×10^{-14} moles/sec. From Fatt's calculation ('50) it would appear that this order of inward acetylcholine$^+$ flux is required to balance the increase in outward K^+ flux brought on by the reduction in membrane potential.

It is important to point out that in these experiments, acetylcholine was applied for a period of several seconds, and therefore the total amount delivered to the end-plate membrane was much greater than the quantity normally made available by a single nerve impulse. From this, and also from the work of Fatt and Katz ('51), it appears that during neuromuscular transmission, the depolarization due to the penetration of positive charge carried by acetylcholine$^+$ is insignificant.

It is also important to emphasize that the single fiber technique is essential in experiments of the type just discussed. The use of a whole muscle, with extracellular electrodes for measuring depolarization at an "end-plate focus" as Fatt

('50) has done, involves certain difficulties. One complication is that the end-plates are not localized in a discrete zone. Another is that the magnitude of the potential difference appearing in the extracellular fluid will depend on its resistance per unit length of muscle. The substitution of sodium free Ringer for ordinary Ringer involves approximately a 20-fold increase in extracellular fluid resistance. Demarcation potentials obtained with the muscle first in one solution and then the other cannot be compared without taking the resistance change into account. In practice, however, the correction is difficult to apply to a multi-fiber preparation.

From the experiments on single fibers, one is led to the conclusion that acetylcholine causes a reduction in the potential of the end-plate membrane *primarily* by increasing its permeability for sodium ions. It is the inward penetration of sodium ions which provides practically all of the positive charge required to depolarize the membrane in neuro-muscular transmission. The explanation of the diminution in overshoot and slowing of repolarization which occurs at the end-plate when an action potential is initiated via the nerve must therefore be based on some relation between acetylcholine and sodium transfer. The following represents an attempt at such an explanation.

It is assumed that in the absence of acetylcholine, the receptive area of end-plate membrane behaves like the remainder of the muscle fiber membrane. Further, that this behavior follows the principles which have been outlined by Hodgkin and Katz ('49), and Hodgkin, Huxley and Katz ('49). At membrane potentials between $+90$ and $+45$ mV. the permeability of the membrane to sodium ions is low. When the membrane potential is reduced beyond the critical level, the sodium permeability rises rapidly, an increase in Na^+ ion current occurs, the membrane potential is rapidly reduced and it finally reverses in sign. This is followed by a reduction in sodium permeability and therefore sodium current.

It is postulated that the reaction of the resting end-plate membrane with acetylcholine causes an increase in its per-

meability to sodium ions to the point that appreciable sodium ion entry becomes possible when the membrane potential is at the resting level. Further, compared with the normal membrane, the sodium ion current will be greater at all values of membrane potential between + 90 and 0 mV. But at membrane potentials between 0 and — 51 mV., the effect of acetylcholine is to decrease the sodium current below that of the normal membrane. It is this relative reduction of sodium current at the crest of the action potential which is responsible for the decrease in overshoot. The slowing in the repolarization of the membrane may be explained by assuming that the sodium permeability does not diminish as rapidly as it does in the normal membrane, but that the fall is delayed by the presence of persisting acetylcholine. In other words, during the falling phase of the action potential the sodium current is higher than would normally be expected.

One of the ways in which acetylcholine$^+$ ions might influence sodium permeability is by entering into combination with sodium carriers thereby reducing the residual negative charge of these molecules. The effect of this is to alter the distribution of carrier molecules in the resting membrane so that an appreciable increase in sodium entry becomes possible at all values of membrane potential between + 90 mV. and zero. However, at membrane potentials between zero and — 51 mV., the partial neutralization of carrier charge would act to reduce sodium entry below normal levels.

From the preceding hypothesis one would predict that the membrane resistance at the end-plate measured at a time that a neurally initiated spike is at its peak would be higher than the membrane resistance at a point distant from the end-plate measured at the time that an electrically initiated action potential is at its peak. From the "short circuit" hypothesis one would predict that the opposite relation would be found. As yet there is no data available which bears on this critical point.

SUMMARY

1. A technique useful in the study of resting and action potentials of single muscle fibers at the region of the end-plate is presented.

2. A typical record of a neurally initiated action potential made with an internal electrode placed at the center of the end-plate is shown.

3. Evidence is presented to show that following the arrival of a nerve impulse, the initiation of activity of the end-plate membrane is signalled by a reduction of membrane potential; no preceding hyperpolarization of this membrane is apparent.

4. The characteristics of action potentials neurally initiated at the end-plate, and the characteristics of action potentials propagated through zones distant from the end-plate are given. A comparison of certain salient features of these groups of action potentials shows that for the neurally initiated action potentials there is an increase in the maximum rate of rise amounting to 9%, a diminution of overshoot amounting to 14 mV., and a reduction in the average rate of repolarization.

5. The muscle fiber membrane reaches a critical state when the potential across it is reduced to + 47 mV.

6. Evidence is presented to show that the depolarization of the end-plate membrane produced by the application of acetylcholine depends *primarily* on the presence of sodium ions in the extracellular fluid.

7. It is suggested that the peculiarities in the shape of neurally initiated action potentials arise from alterations of the end-plate membrane characteristics produced by the reaction of this membrane with acetylcholine. Further, the principal action of acetylcholine is to cause a relative increase in the sodium permeability of the end-plate membrane when its potential lies between + 90 and 0 mV., and to cause a relative decrease in its sodium permeability at membrane potentials between zero and — 51 mV.

ACKNOWLEDGMENTS

I wish to express my gratitude to Hoffmann-La Roche, Inc., for generous financial aid. I am also greatly indebted to Mr. J. T. Alexander for valuable suggestions and assistance given during the course of this work.

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THE EFFECT OF IONIZING RADIATIONS UPON THE RESPIRATION AND OXIDASES OF THE POTATO TUBER [1]

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FOUR FIGURES

There have been few recent and critical investigations dealing with the effect of ionizing radiations upon higher plant metabolism. The work that has been done was either concerned with microorganisms (Giese, '42, '47) or with hormonal effects on higher plants (cf. p. 38 of the review by Sparrow and Rubin, '52). Therefore, it was the purpose of the following experiments to determine the effect of radiations upon the metabolism of the potato tuber, as expressed by its respiratory rate, and by the activity of its oxidases.

MATERIALS AND METHODS

The tubers used in these experiments were of the Irish Cobbler variety and were approximately 1½″ in diameter. It was necessary to use such small tubers because of the size restrictions imposed by the irradiation techniques used. They

[1] I would like to thank Dr. Arnold H. Sparrow for his encouragement and help given during the course of this work, as well as Mr. Eric C. Christensen who provided valuable assistance in the procuring of materials and in the accomplishment of the irradiation. Also, Dr. Raymond Klein provided valuable discussion and materials for the pursuit of certain of these experiments.

were stored at room temperature and in all cases were used less than 60 days after being harvested in July. In computing the tuber volumes for the purpose of calculating the flask constants in respirometric experiments, a density of 1.12 was used.

Respirometric experiments were carried out using modified Warburg vessels of approximately 80 ml capacity. The tubers were equilibrated to the temperature of the water bath (28°C.) for 60 minutes after which the CO_2 output was measured for 60 minutes by the direct method. Oxygen consumption was then measured at 10 minute intervals for 60 minutes. It was assumed that the equilibration period was sufficient since linear rates were obtained. At least 6 tubers were used in each experiment and the values for the Q_{O_2} [2] and Q_{CO_2} which are reported represent the average of these samples.

Cytochrome oxidase and tyrosinase activity were determined using the manometric techniques described by Goddard and Holden ('50). Warburg vessels of 17 ml capacity were used in these experiments, and were shaken at a rate of 120 oscillations per minute at 28°C. The enzyme preparations were made by chopping the potatoes with an equal volume of cold 0.1 M sodium veronal buffer, pH 7.1, in a Waring blendor for 5 minutes. This "brei" was filtered through cheese cloth and the filtrate centrifuged at 500 × gravity for 10 minutes. In order to measure cytochrome oxidase activity an aliquot of this supernatant was diluted with an equal volume of 0.1 M phosphate buffer, pH 7.1, and used in the respirometric determination. Tyrosinase activity was measured after diluting another aliquot of the supernatant with 20 volumes of 0.1 M phosphate buffer, pH 6.8.

Irradiation with gamma rays was accomplished by exposure to Tantalum^{182} sources delivering about 80,000 to 200,000 r per hour. The tubers were handled as described by Manowitz ('51): an aluminum tube about 12″ in length and 1½″ in diameter was used as a container for the potatoes during exposure.

[2] Q_{O_2} as used in these experiments is equivalent to mm^3O_2/hour/mg fresh weight; Q_{CO_2} is equivalent to mm^3CO_2/hour/mg fresh weight.

Calibration of the source was accomplished by means of the techniques outlined in Weiss ('52). Doses of 100 r were obtained by exposing the tubers to a Cobalt^{60} source in the Brookhaven "Gamma Field" described by Sparrow and Singleton ('52).

The x-ray source was a G.E. Maxitron 250 with a beryllium window tube, delivering 50,000 r at 23 cm Target to Subject Distance (TST) at a rate of 1428 r per minute. The Half Value (HVL) was 0.2 mm of copper, and the dosage was measured in air with a Victoreen Integron dosimeter. During irradiation, the potatoes were rotated in order to insure equal penetration to all tissues.

RESULTS

The respiratory gas exchange of tubers was determined immediately before irradiation and periodically thereafter. The effect of such treatment upon the Q_{O_2} of potato tubers is shown in figure 1. In all cases there was an imme-

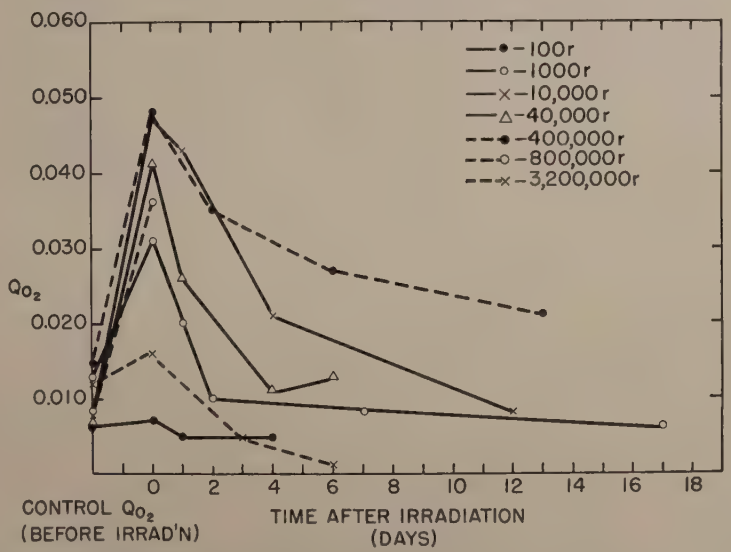

Fig. 1 The effect of gamma-irradiation upon the oxygen uptake of potato tubers. (Q_{O_2} calculated as mean of 6 tubers.)

diate increase (0- day reading) in oxygen uptake which was approximately fivefold at dosages between 10,000 to 400,000 r and two- to threefold at 1000 r and 3,200,000 r. There was a slight increase at 100 r but a "chi-squared" test showed that this was not significant. At all dosages except the highest, the Q_{O_2} decreased after the first day and, in most cases, gradu-

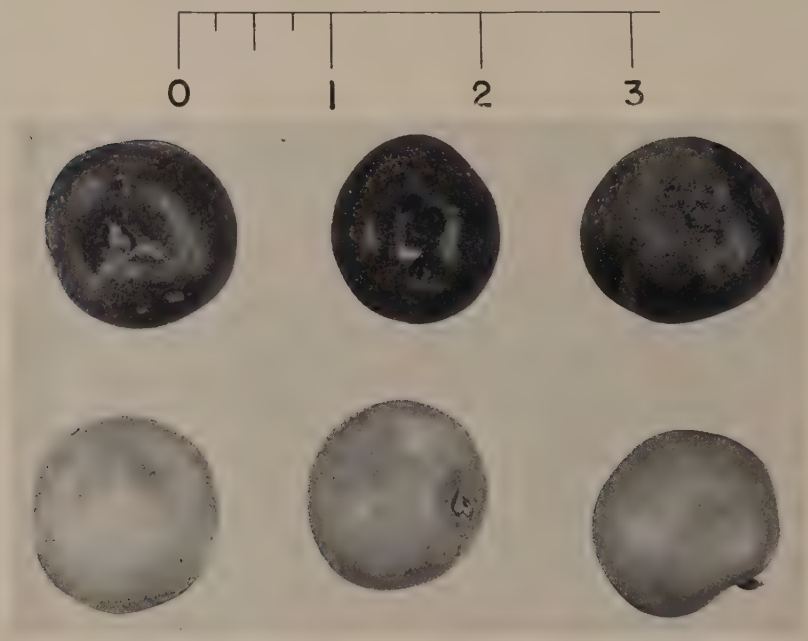

Fig. 2 The appearance of potato tubers 72 hours after receiving 3,200,000 r gamma rays. (Scale in inches.) Top row contains the treated tubers while the bottom contains untreated controls.

ally returned to the control rate or to a rate slightly below this. At 3,200,000 r, however, the Q_{O_2} at the end of 6 days after irradiation was low enough to suggest the total disruption of the potato's metabolism. This respiratory change was accompanied by the gradual blackening and softening of the tuber, as is shown in figure 2. Such visible changes began immediately after irradiation with the appearance of bluish-black spots over the epidermal surface as well as with a

slight darkening of the entire tuber. Samples from the cortex, epidermis, and vascular region of these tubers were sectioned immediately after irradiation but no evidence of necrosis or other tissue injury was discovered.[3]

The effect of irradiation upon the Q_{CO_2} of tubers is shown in figure 3. As in the case of the Q_{O_2}, the Q_{CO_2} was also increased immediately after irradiation, although the magnitude of this

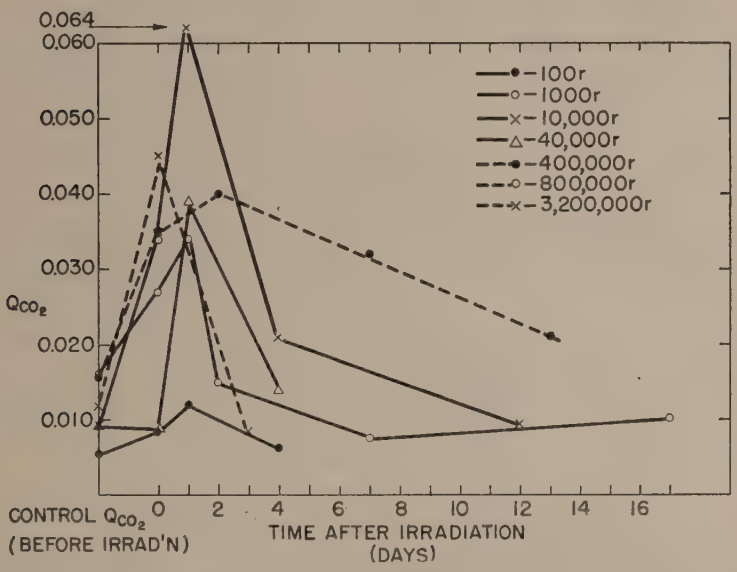

Fig. 3 The effect of gamma-irradiation upon the output of carbon dioxide by potato tubers. (Q_{CO_2} calculated as mean of 6 tubers.)

increase was usually much smaller than in the former case. It was only during the second day after irradiation that the peak of the increase in CO_2 production was reached. This is very evident when the data are plotted as in figure 4. Thereafter, however, this rate decreased more or less parallel to the Q_{O_2}. In the case of the 100 r and the 3,200,000 r treated tubers the Q_{CO_2} increased more than did the Q_{O_2}. In the latter case there was

[3] The sectioning reported here was performed by Dr. James Gunckel and Miss Ielene Morrow who also checked the author's observations of the irradiated tissue.

about a threefold differences between these increases but in the former the difference was only about one-third of this. In order to determine whether ammonia was being evolved by tubers irradiated with 3,200,000 r, 0.5 ml of 10% HCl was used in the side-arm instead of water but there was no difference in the amounts of gas evolved. Ammonia analyses run in the Van Slyke apparatus were likewise negative. Moreover,

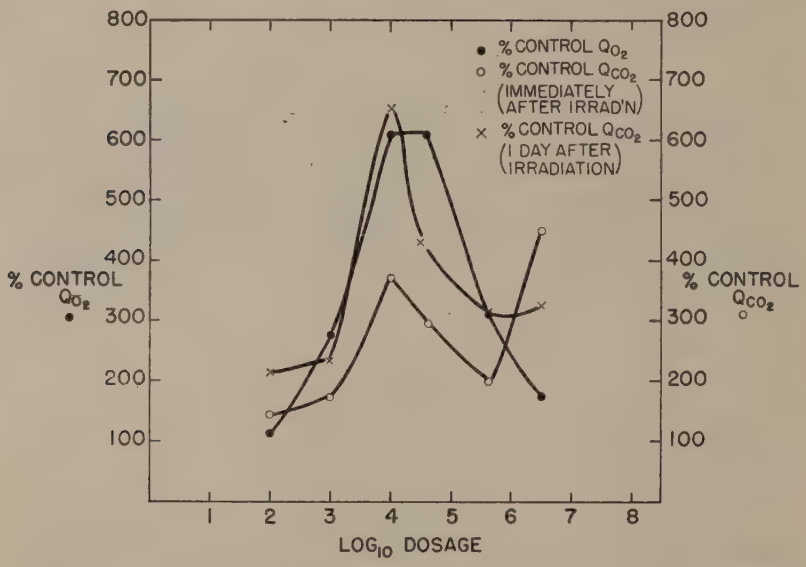

Fig. 4 The effect of gamma-irradiation upon the Q_{O_2} and Q_{CO_2} of potatoes as a function of dosage and time after irradiation.

a mixture of $AgNO_3$ and HNO_3 was used in the side-arm in order to trap ethylene in case this gas was being produced but again negative results were obtained. The pH of "breis" prepared from control tubers and those irradiated with 3,200,000 r was measured by the use of a Beckmann pH meter in order to determine whether such changes could account for the increase in the amount of CO_2 liberated by the treated tubers. About 50 ml of potato tissue were ground for one minute in a Waring blendor and filtered through cheese cloth. The pH of both control and treated tubers was 6.0.

Tubers were also exposed to X-irradiation and, in general, the same results were obtained as in the case of those that were irradiated with gamma rays. These results are given in table 1.

TABLE 1

The effect of x rays upon the respiration of potato tubers

DOSAGE (r)	CONTROL		DAYS AFTER IRRADIATION			
			0		2	
	Q_{O_2}	Q_{CO_2}	Q_{O_2}	Q_{CO_2}	Q_{O_2}	Q_{CO_2}
50,000	0.011	0.011	0.042	0.022	0.034	0.046
500,000	0.006	....	0.040	0.039	....	....

Finally, the cytochrome oxidase and tyrosinase activities of irradiated tubers were measured with the results shown in table 2. No significant effect upon the activity of these enzymes could be demonstrated immediately after irradiation

TABLE 2

The effect of gamma (γ) and x rays upon the cytochrome oxidase and tyrosinase activity of potato tubers

DOSAGE (r)	TYPE OF RADIATION	CYTOCHROME OXIDASE ACTIVITY	TYROSINASE ACTIVITY
		mm^3 O_2/30 minutes	*mm^3 O_2/30 minutes*
Control	....	119, 108	225
40,000	γ rays	114, 117	220, 200
400,000	γ rays	100	200
3,200,000	γ rays	122	90, 110
50,000	x rays	94	212

except in the case of the tubers exposed to 3,200,000 r. At this dose, there was about a 50% decrease in tyrosinase activity, although cytochrome oxidase activity was relatively unimpaired.

DISCUSSION AND CONCLUSIONS

It has been demonstrated that respiratory gas exchange in potato tubers is markedly enhanced by dosages of gamma irradiation as low as 1000 r. The effect upon the Q_{O_2} is almost immediate in that increases from two- to sixfold were measureable several hours after the tubers were irradiated. There-

after, a progressive decrease in the oxygen uptake occurred. Similar increases in the Q_{CO_2} were also noted except that the peak of this increase occurred on the first day after irradiation. After this, the Q_{CO_2} decreased in a manner parallel to the Q_{O_2}.

Previous work upon the metabolic effects of radiations on higher plants has shown that such treatment increases the rate of CO_2 production by radish seeds and seedlings (Redfield and Bright, '22; Chesley, '34). On the other hand, Chesley cites several references wherein no increase of this kind could be observed, while Johnson ('26) also found that the CO_2 production of seeds and young seedlings of *Helianthus* produced less CO_2 as a result of X-irradiation.

The literature bearing on the metabolic response of animals to irradiation also shows several well-established cases of increases in gas exchange (cf. Wynd and Reynolds, '35). For example increases in the oxygen uptake, as a result of X-irradiation, were noted in frog skin (Williams and Sheard, '32), fowl erythrocytes (Frankenthal and Back, '44), grasshopper eggs (Tahmisian, '49), and in rats (Kirschner, Prosser, and Quastler, '49). That such increases can also be observed in homogenates was shown by Richmond ('51) using bone marrow and by Kunckel and Phillips ('52) who used rat liver. However, divergent results were obtained by Hevesy and Forssberg ('51) who found that X-irradiated mice exhaled less CO_2 than did controls; moreover, Klein ('52) also failed to get any increases in the oxygen uptake of erythrocytes from X-irradiated ducks.

Such discrepancies have also arisen during studies on the effect of radiations on microorganisms (cf. brief review in Giese, '42). A possible explanation for some of these differences has been suggested by Giese ('42, '47) as a result of his work on yeast. Ultra-violet irradiation of these organisms was found to increase the rate of endogenous respiration more than tenfold although the rate of exogenous respiration was decreased under the same conditions. Similar results were recently obtained with *Chlorella* (Redford and

Myers, '51) although the increase in the endogenous rate was only transitory.

Since the present experiments were performed with freshly harvested potato tubers these were probably in the dormant state. This was confirmed by germination tests which showed that unirradiated tubers used in these experiments developed shoots under greenhouse conditions only after 4–6 months. It seems possible, although no confirmatory experiments have been carried out, that more rapidly metabolizing potatoes will show different responses to irradiation than did these.

Attempts have been made to explain the mechanism by which respiratory increases arise due to irradiation but these are largely speculative. Tentatively, a reasonable explanation might assume the production of a substance or substances which permit the cell to metabolize its available food reserves more rapidly than could be done before irradiation. That such changes may be connected to phosphate metabolism is suggested by Giese's ('47) results which showed that dinitrophenol (DNP) accelerated the rate of endogenous metabolism in yeast as well as synergistically increasing the oxygen uptake of irradiated cells. Further experiments along these lines are now being conducted in this laboratory in an attempt to explain the results obtained with potatoes.

It should also be noted that the respiratory systems of potatoes are much more resistant to irradiation than is the capacity for growth as judged by germination. Sparrow and Christensen ('50) have showed that exposure to 4800 r gamma irradiation almost entirely prevented the germination of potato pieces whereas the respiration of the tubers used in the present experiments continued for indefinite periods after almost 100 times this dosage. The results of germination tests during the present experiments largely confirm the previous findings.

The maintenance of cytochrome oxidase activity at approximately the same level after receiving all the dosages administered during these experiments suggests that the mechanism of radiation damage to growth must be sought elsewhere.

Similarly, tyrosinase activity remains constant until tubers are exposed to 3,200,000 r so that changes in this enzyme's activity cannot be directly connected with the observed changes in metabolic rate and germinability. However, the blackening of the tubers given 3,200,000 r, accompanied by the decrease in tyrosinase activity, suggest a possible relationship. Since the products of this enzyme's activity are known to poison it (Nelson and Dawson, '44), it is conceivable that the blackening of the tubers is due to the accumulation of these products with subsequent injury to the enzyme.

SUMMARY

1. Potato tubers (Irish Cobbler variety) show increases in Q_{O_2} up to 600% as a result of gamma ray irradiation over a wide range of dosages.

2. Carbon dioxide evolution is also increased although the peak of this increase occurs 24 hours after that of the oxygen uptake.

3. Cytochrome oxidase and tyrosinase activities were not affected by the dosages used in these experiments except that the latter enzyme lost half of its activity at 3,200,000 r.

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THE EFFECTS OF POTASSIUM ON BACTERIAL LUMINESCENCE INTENSITY WITH REFERENCE TO THE TEMPERATURE AND PRESSURE CONDITIONS[1]

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THREE FIGURES

In a preceding paper (Schneyer, '51) it was pointed out that low concentrations of calcium and phosphate salts together (Ca 10 mM.; PO_4 2.5 mM.), but not separately, increase the intensity of bacterial luminescence throughout the viable temperature range and that this effect may be augmented by the application of high hydrostatic pressure (400 atmospheres).

In the present study, the effects of potassium salts on bacterial luminescence intensity have been investigated. Johnson and Harvey ('38) have reported an inhibitory effect of potassium on this system at temperatures near the optimum for luminescence. The present work corroborates the findings of Johnson and Harvey for this temperature range. It is now found, however, that the inhibiting effect of potassium decreases with a reduction in temperature from any point above the optimum and, in fact, becomes a stimulating effect at still lower temperatures. This stimulating effect resembles the Ca-PO_4 effect in that it is pressure sensitive. Furthermore,

[1] This investigation was carried on as part of a project at New York University directed by Dr. Dugald Brown and aided by a grant from the Cinchona Products Institute, Inc.

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like Ca and PO_4, potassium slows the irreversible inhibition of light intensity by high temperature.

METHOD

Photobacterium phosphoreum was cultured at 8°C. according to the methods outlined in a previous paper (Schneyer, '51). Bright cultures of the bacteria were suspended in isotonic buffered sodium chloride in the case of the controls or in buffered sodium and potassium chlorides in the case of the experimentals, each at pH 7.3. Monobasic and dibasic sodium phosphate (M/8) or sodium cacodylate (M/10) were used as the buffer salts. Measurements of relative light intensity

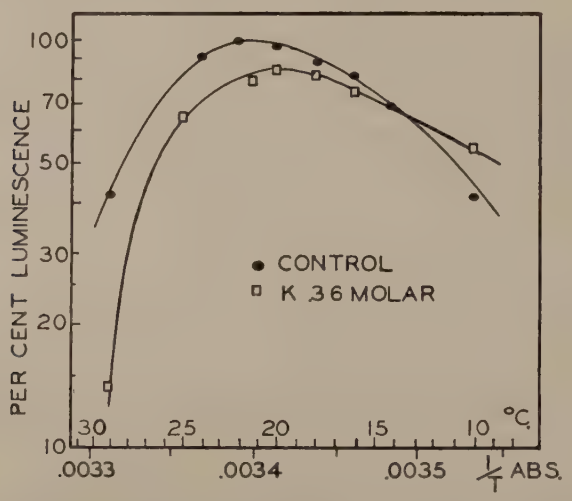

Fig. 1 Relation between $\log_{10}$ luminescence intensity and temperature for control and KCl-containing bacterial suspensions. Intensity is expressed as per cent of the maximum value of the control which is designated 100.

were made with the photomultiplier photometer over the range of linear response as described in a previous paper (Schneyer, '51). Pressure was applied by the use of the pressure pump and bomb described by Brown (Brown, Johnson and Marsland, '42).

RESULTS

Effect of changes in temperature

The data plotted in figure 1 show the behavior of luminescence intensity between 10 and 30°C. for a control suspension of luminous bacteria and for one containing 0.36 *M* potassium chloride, each at pH 7.3 (cacodylate buffer). The inhibiting effect of potassium reported by Johnson and Harvey for temperatures near the optimum (22°C.) is clearly shown in figure 1. In addition, it is evident that the inhibition increases markedly as the temperature is raised beyond the optimum.

TABLE 1

Action of temperature on the potassium effect

NO.	MOLAR K	BUFFER pH 7.3	LOW TEMPERATURE °C.	I	% In [1]	MODERATE TEMPERATURE °C.	I	% In [1]	HIGH TEMPERATURE °C.	I	% In [1]
	0.00	Phosphate		18			78.8			21	
1			5.0		—71	22.0		13	35.5		52
	0.36	.025M.		31			69.2			10	
	0.00	Phosphate		20			71.7			14	
2			5.0		—75	22.0		11	35.5		43
	0.36	.025M.		35			63.8			8	
	0.00	Cacodyl-		18			43			18	
3		ate	10.0		—28	22.0		21	29.0		45
	0.36	.10M.		23			34			10	

[1] *In* = inhibition (negative value indicates stimulation).

With a reduction in temperature below the optimum, the inhibition of luminescence intensity by potassium decreases rapidly. From the data represented in figure 1, it is apparent that no inhibition is observable at 13°. Below 13° the potassium caused an augmentation of luminescence intensity which reached a value of 28% at 10°C.

The data presented in table 1 show these effects of potassium in greater detail. In a representative case, 0.36 *M* KCl caused an increase in luminescence intensity of 71% at 5°C.

At 22°, the same bacterial suspension was inhibited 13% by the KCl, and at 35.5° the inhibition had increased to 52%.

The presence of potassium reduces the irreversible heat inactivation of luminescence even though the inhibiting effect of potassium is increased at high temperatures, as shown in table 2. In each experiment outlined in table 2, the luminescence intensities of the control and experimental suspensions were measured at 23°, a temperature close to the optimum for luminescence, and the temperature was then raised rapidly to 31°C. At this temperature, irreversible inactivation

TABLE 2

The effect of potassium on the denaturing action of high temperature

NO.	MOLAR K	BUFFER pH 7.3	INTENSITY AT 23°C. (I_1)	INTENSITY AT 31°C. (I_2)	RETURN TO 23°C.		
					Intensity (I_3)	Per cent of I_1	Protection
							%
1	0.00	Cacodylate	61.4	14.5	36.4	61	12
	0.37	Cacodylate	50.0	8.5	36.4	73	
2	0.00	Phosphate	58.0	27.0	31.0	54	17
	0.37	Phosphate	59.8	26.0	42.8	72	
3	0.00	Cacodylate	95.0	42.0	48.0	51	26
	0.12	Cacodylate	92.0	35.0	70.0	77	
				29°C.			
4	0.00	Cacodylate	95.0	42.0	78.0	82	18
	0.36	Cacodylate	75.0	14.0	77.0	100	

proceeds rapidly (Johnson, Eyring, Steblay et al., '45). The temperature of the suspensions was returned to 23° and the intensities were again measured. The per cent irreversible temperature inhibition was calculated. The results show that, in the presence of potassium, the irreversible inhibition by heat was about 20% less than in the absence of potassium.

The effects of temperature on the action of potassium are readily reversible. Thus, the data given in table 2 show that the increase in inhibition by potassium which occurs with an increase in temperature may be completely reversed by again lowering the tempreature. The data from experiment no. 4

in table 2, from which figure 1 is plotted, show an inhibition by potassium at 23° of 21%. At 29°, the inhibition had increased to 67%. Lowering the temperature again to 23° reduced the inhibition to 1%. The reduction in the inhibition by potassium at 23° after heat treatment is due to the effect of potassium on the irreversible inactivation by heat.

The results of two experiments on the reversibility of the low temperature stimulation by KCl are given in table 3. In each experiment the effect of potassium was small at the optimum temperature. When the temperature was lowered to 5°C., the potassium caused a stimulation of about 75%; and

TABLE 3

Reversibility of the low temperature potassium effect (phosphate buffer)

NO.	MOLAR K	22.0°C.		5.0°C.		22.0°C.		% REVERSIBILITY
		I	% Inhib.[1]	I	% Inhib.[1]	1	% Inhib.[1]	
1	0.00M.	78.2	10	18.0	—71	70.2	4	90
	0.36M.	71.0		31.0		67.8		95
2	0.00M.	71.7	—11	20.0	—75	72.0	—6	100
	0.36M.	80.0		35.0		76.2		96

[1] Positive value represents inhibition; negative value, stimulation by potassium.

upon return to 22°, the stimulation was reduced to about 4% or a small inhibition appeared, depending upon whether potassium had originally caused a stimulation or an inhibition at that temperature. A reversibility of about 95% of the low temperature effect of potassium was found.

Effect of compression at low temperature

At temperatures below the optimum for luminescence, pressure reduces light intensity in bacterial suspensions to which potassium has and has not been added. However, pressure does not reduce the intensity of luminescence to the same extent in the two cases. Figure 2 shows the effects of the application of hydrostatic pressures up to 6000 lbs./sq. in. to

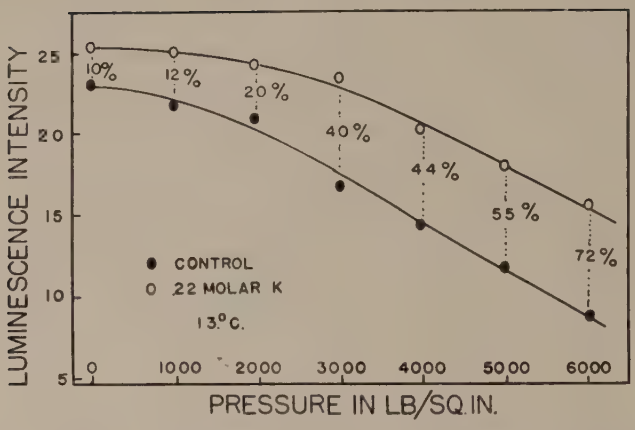

Fig. 2 The effect of hydrostatic pressure on the augmentation of luminescence intensity by potassium at 13°C. Intensity values are in arbitrary units and per cent augmentation at each 1000 lbs./sq. in. interval is indicated.

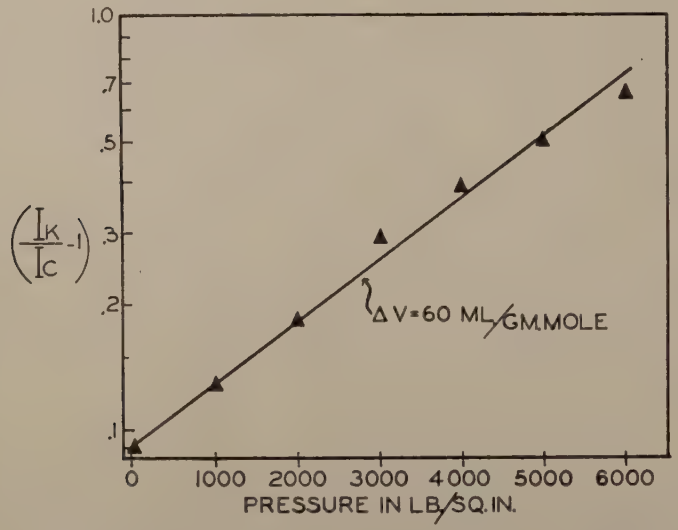

Fig. 3 Analysis of the effect of pressure on the action of potassium at 13°C. Data are from experiment plotted in figure 2. I_c represents intensity of control; I_k represents intensity with 0.22 *M* KCl. Calculated volume change is — 60 ml per gram mole.

a control suspension and to one containing 0.22 *M* KCl, both at 13°C. The luminescence intensity of the control suspension was inhibited by 60% of its intensity value at atmospheric pressure by the application of a pressure of 6000 lbs./sq. in., while the intensity of a suspension containing 0.22 *M* KCl was inhibited only 38% by the same pressure. The effect of pressure on the stimulation by potassium at temperatures below the optimum is to increase the stimulation. In the experiment cited in figure 2, the stimulating effect of potassium at 13° increased from a value of 10% at atmospheric pressure to a value of 72% at a pressure of 6000 lbs./sq. in.

Application of the theory of absolute reaction rates (Glasstone, Laidler and Eyring, '41) to the pressure data permits estimation of the molecular volume change involved in the augmentation of luminescence intensity by potassium. As shown in figure 3, a decrease in volume of 60 ml per gram mole is indicated.

DISCUSSION

The results indicate that potassium acts at more than one locus in the luminescent system, the dominant action being dependent on the temperature range chosen.

At temperatures above 30°C., potassium slows the irreversible inactivation of the light emitting system. This effect of potassium is similar to that caused by calcium and phosphate at high temperature (Schneyer, '51) and is evident in spite of the very appreciable inhibition of luminescence intensity caused by potassium within this temperature range.

The present investigation has not been directed primarily at elucidating the inhibitory action of potassium first reported by Johnson and Harvey. In view of the fact, however, that the data given here show clearly that this inhibition increases with a rise in temperature and is largely reversible with a reduction in temperature, it appears possible that the inhibiting effects of potassium, like those of urethane (Johnson, Eyring and Williams, '42) and quinine (Johnson and Schneyer, '44), are due to an action on the reversible denaturation process which increases its rate.

In view of the large negative volume change which characterizes the stimulatory action of potassium at low temperatures, it is reasonable to assume that some action on a protein is involved. While this stimulation by potassium may be due indirectly to reversal of an enzyme inhibition, the magnitude and direction of the volume change accompanying the augmentation process and the low temperature at which the augmentation occurs are suggestive of a direct action on a native enzyme which modifies its molecular volume and, hence, its activity.

In this connection, it is of interest to note that as a result of a study of activation energies of a variety of enzyme reactions, Sizer ('43) has suggested that native enzymes may shift from one configuration to another, particularly at low temperatures. Kavanau ('50) has recently suggested that at low temperatures "reactive enzyme particles" are converted to a catalytically inactive condition "in which the active centers either lose their specific configuration or are no longer exposed to the substrate."

It appears, therefore, that, while this has not been proved for bacterial luminescence, native enzymes may exist in multiple forms, each differing in regard to stability and activity. The effects of hydrostatic pressure on the augmentation of luminescence by potassium at low temperatures is suggestive of the existence of two forms of a native enzyme which is pace-setting for the luminescent process. The effects of pressure make it necessary to assign a smaller volume to the more active molecular form. Modification by potassium of an equilibrium between these two forms (in favor of the more active form) would satisfactorily account for the augmentation of luminescence by potassium at low temperatures.

SUMMARY

The effects of potassium chloride on luminescence intensity of P. phosphoreum have been studied under varied conditions of temperature and pressure. It has been found that potassium acts at more than one locus in the luminescent system;

the dominant action is dependent on the temperature range chosen. At temperatures above 30°C., potassium slows the irreversible inactivation of the system by heat. At temperatures near the optimum for luminescence (22°C.), potassium decreases luminescence intensity as reported by Johnson and Harvey. At temperatures below the optimum, potassium may increase the intensity of luminescence. This action is increased by the application of high hydrostatic pressure and appears to be related to a shift in the configuration of a native protein.

ACKNOWLEDGMENT

I wish to thank Dr. Dugald Brown, in whose laboratory this work was done, for helpful suggestions during the course of the experimental work.

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VELOCITY OF CONDUCTION IN FROG NERVE AS MODIFIED BY APPLIED CURRENTS, KCl AND $CaCl_2$ [1]

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TWELVE FIGURES

No quantitative values are to be found in the literature concerning the velocity of conduction in nerve as modified by applied currents, probably because the usual bipolar method of applying currents results in large gradients of membrane current distribution along the nerve and consequently the velocity of conduction in the polarized region is very non-uniform. Very little has been added to the qualitative conclusions of Rutherford (1868) that the velocity was reduced both under the anode and the cathode for large currents and increased, or not changed under the cathode for small currents applied for not too long a time. Rutherford refers to the work of von Bezold, who in 1861 showed that large anodal or cathodal applied currents reduced the velocity. The results of Rutherford were confirmed oscillographically by Bishop and Erlanger ('26). Schmitz and Schaefer ('33) showed that near an anode there is a transient block, or a period during which the velocity is reduced below its steady state value.

Interest in the effect of high potassium on the velocity of conduction as modified by applied currents arises from the

[1] Preliminary report: Fed. Proc., *9:* 124 (1950). This work comprised a portion of a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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well known ability of anodal polarization to relieve a conduction block due to many agents. Woronzow, in a series of papers (see Lorente de Nó, '47a, b) reported that anodal polarization could relieve a block due to KCl, RbCl, CsCl, NH_4Cl, NaOH and $ZnCl_2$. He also reported that block due to $CaCl_2$, $BaCl_2$ and $MgCl_2$ was relieved by cathodal currents. Although the list of anodally relieved blocking agents has grown to include Ether, Eserine, Iodoacetamide, Iodoacetic Acid, Cyanide, Cupric Ions, Veratrine and Faradization (Lorente de Nó, '47b), as well as Cocaine, x rays, Ethyl Alcohol (Gallego, '48) and Anoxia (see esp. Lorente de Nó, '47a, b), no other substances which produce block relieved by cathodal polarization have been reported.

Some of the anodally relieved blocking agents lower the membrane potential. Unfortunately, very few investigators have measured the changes in membrane potential at the time of block. One would tend to list IAA as a substance which lowered the membrane potential, but Bishop ('32) found that at the time of block, IAA (1/2000) had actually increased the potential by a small amount. The same has been reported for DFP and Eserine (Toman et al., '47) and Cocaine (Bishop, '32).

To say that the anodal polarization relieves the block because it increases the membrane potential is rather tautological. It will be shown that in anodal relief of potassium block the nerve will conduct impulses, but not at the normal velocity.

In order to measure the effect of applied currents on the conduction velocity what one would like to have is a relatively long region of nerve over which the applied membrane current is constant. This has been done with squid nerve by the introduction of micro electrodes into the axoplasm (Hodgkin, Huxley and Katz, '49; Marmont, '49) but such a procedure is not possible on nerves of small diameter. The author has developed a method for achieving this result with external electrodes.

The mathematical analyses associated with the development of this method are presented elsewhere (Taylor, '52). The basic idea is that the membrane current will be constant throughout most of any large enough length of nerve over which the longitudinal applied current density is increasing linearly with distance, provided only that the properties of the nerve do not change with distance.

The problem of shaping a two dimensional region such that along some straight line of current flow the current density increases linearly will be shown directly to have a unique solution. Cremer achieved a close approximation with his "Winkelrinne" and Keil, in Cremer's laboratory constructed a great variety of differently shaped chambers and measured the current distributions (Cremer, '29, p. 274). One of the shapes which Keil used was similar to that employed in the present research.

A theoretically correct chamber shape will be derived. However, other factors are involved, such as ease of construction, distortion due to the presence of the nerve, etc. The actual shape used is thus a compromise.

METHODS

1. Chamber shape

The objective for the two dimensional electrode is to provide a region over which the current density increases linearly along some straight line of current flow. This can be derived in the following way:

In any two dimensional region which contains no sources or sinks of current, the equations of current flow and constant voltage are solutions of Laplace's equation, which in rectangular coordinates (x, y) is

$$\frac{\partial^2 V}{\partial x^2} + \frac{\partial^2 V}{\partial y^2} = 0.$$

It can be shown that the real and imaginary parts of any analytic function of the complex variable $z = x + jy$ ($j = \sqrt{-1}$) are conjugate solutions of Laplace's equation. Thus

these two functions will be the lines of current flow and constant potential for *some* boundary value problem. The present problem is to find the boundary conditions such that the current distribution has the property discussed above.

Let:

$U(x, y)$ be the potential function (lines of constant voltage)

$V(x, y)$ be the stream function (lines of current flow)

$i(x, y)$ be the current density at the point (x, y)

t be the resistivity of the medium in ohms

$W = U + jV$.

For convenience, let the current density and the voltage at the origin be zero and let the x axis be the line along which the current density is to increase linearly. The problem then becomes: what is the analytic function $W = U + jV$ such that $i(x, O) = kx$ and $V(x, O) = \text{constant}$?

There is no loss of generality in letting $V(x, O) = 0$.

A well known relation in potential theory (Smythe, '39, p. 229) is

$$i(x, y) = \frac{1}{t}\left|\frac{\partial W}{\partial z}\right|.$$

Thus:

$$i(x, O) = \frac{1}{t}\left|\frac{\partial W(x + jO)}{\partial z}\right|,$$

and since $V(x, O) = 0$,

$$\frac{\partial W(x + jO)}{\partial z} = \frac{dU}{dx},$$

we can write

$$i(x, O) = kx = \frac{1}{t}\left|\frac{dU}{dx}\right|.$$

Integrating this gives the expression for U on the x axis, which, since the constant of integration is zero is

$$U(x, O) = \frac{ktx^2}{2} = W(x, O).$$

Now $U(x, O)$ is a function of x, equal to W on the x axis. Clearly

$$W(z) = \frac{ktz^2}{2}$$

is an analytic function, which by the principle of analytic

continuation is the unique continuation of $U(x, O)$ onto the complex plane and is thus the function sought.

With the real and imaginary parts written out,

$$W = \frac{kt}{2}(x^2 - y^2) + jktxy.$$

Thus the lines of constant potential are

$$U(x, y) = \frac{kt}{2}(x^2 - y^2) = \text{constant},$$

and the lines of current flow are given by

$$V(x, y) = ktxy = \text{constant},$$

which are conjugate families of hyperbolae. These functions are plotted for a portion of the (x, y) plane in figure 1.

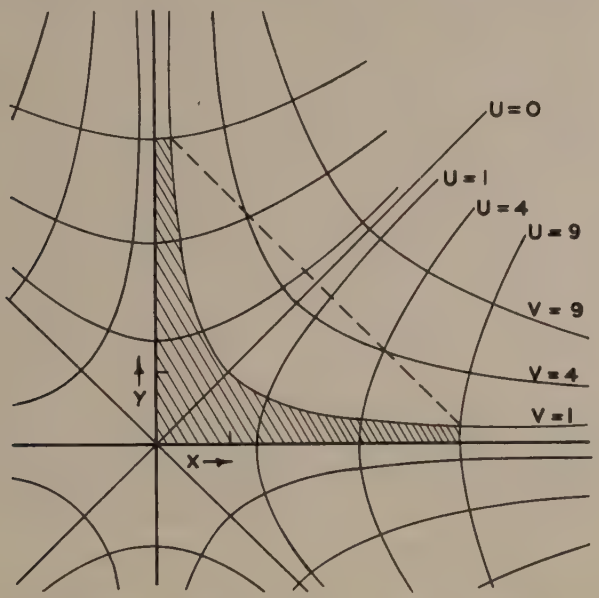

Fig. 1 Plot of potential function $U(x, y)$ and stream function $V(x, y)$ of distribution where current density along x axis is $i(x, O) = kx$. Shaded area represents possible electrode shape. Dotted line forms triangle with portions of x and y axes, which shape was actually used for electrode.

Since $W(z)$ is analytic its value at any point inside a closed region is uniquely determined by its values on the boundary. Therefore, for any region of the (x, y) plane which is bounded by segments of the curves $U =$ constant and $V =$ constant,

the distribution of current in that region will be the same as if it were part of an infinite plane with the distribution W(z).

A boundary of a conducting surface which contains no sources or sinks is necessarily a line of current flow. Many chambers, each containing a region of the x axis could be constructed to obtain the desired result. One of the simplest of these is represented by the shaded area on the graph of figure 1. This region is bounded by the lines $V = 0$, $V = 1$ and $U = \pm 9$. Electrodes would be placed on the line segments $U = \pm 9$.

Such a chamber shape was cut from filter paper and used in the early part of this investigation. However this shape suffers from the difficulty that in certain regions the shape of the curve is very critical. Rather than go through the difficult process of analyzing the effect of small changes in shape, a cut and try method was employed. It was determined that if the line $V = 1$ were straight instead of hyperbolic, the distribution of voltage along the x axis was quite satisfactory and less affected by small errors in cutting the paper.

Thus the electrode consisted of a piece of filter paper cut in the form of an isosceles right triangle with voltages applied at the two acute corners. It was adequately determined that the current density did increase linearly along either edge from the right angle corner and that the presence of the nerve did not disturb this relationship.

As shown in detail elsewhere (Taylor, '52), the membrane current distribution in the fibers of a nerve trunk placed along the x axis would contain a plateau. The membrane current density in the plateau region would be independent of the membrane resistance or the resistance of the epineural sheath, and be directly proportional to the total current applied to the filter paper. The direction of the membrane current would be inward (anodal) if the current direction along the nerve was away from the right angled corner.

A polystyrene chamber was constructed to contain the triangular filter paper electrode, of which figure 2 is a photo-

graph. The tight fitting cover and the calomel electrodes do not appear in the picture. Moist air was circulated through the tubes at either end of the nerve.

J-shaped tubes communicated with the corners of the triangle from below, and with the polarizing circuit through calomel electrodes.

Stimulation and recording was accomplished through electrodes built into the chamber along the edge where the nerve

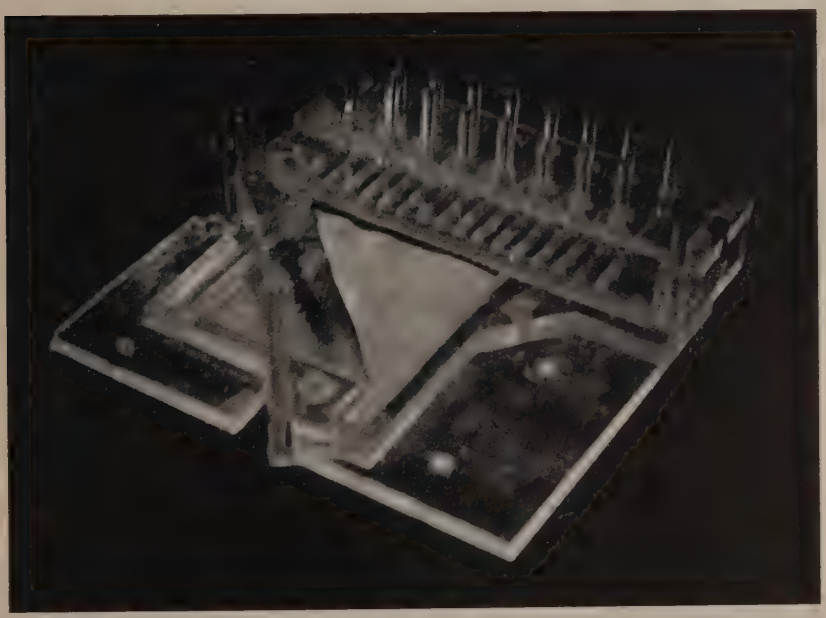

Fig. 2 Photograph of chamber containing triangular filter paper electrode.

was placed. These electrodes, spaced one-half centimeter apart, terminated in small holes through which passed a moist thread. This thread contacted one side of the nerve when it was in place. These electrodes were also connected through calomel electrodes, to a high input impedance amplifier. It was necessary to keep the resistance between leads high so that their presence did not alter the current distribution in the chamber.

2. Electrical equipment

A square pulse generator was used for stimulating the nerves. It was manufactured according to specifications provided by Dr. E. B. Wright and somewhat modified by both of us. The stimulator was used to plate pulse a 5 megacycle oscillator in the isolation unit (fig. 3), essentially like that described by Schmitt ('48). With this amount of isolation,

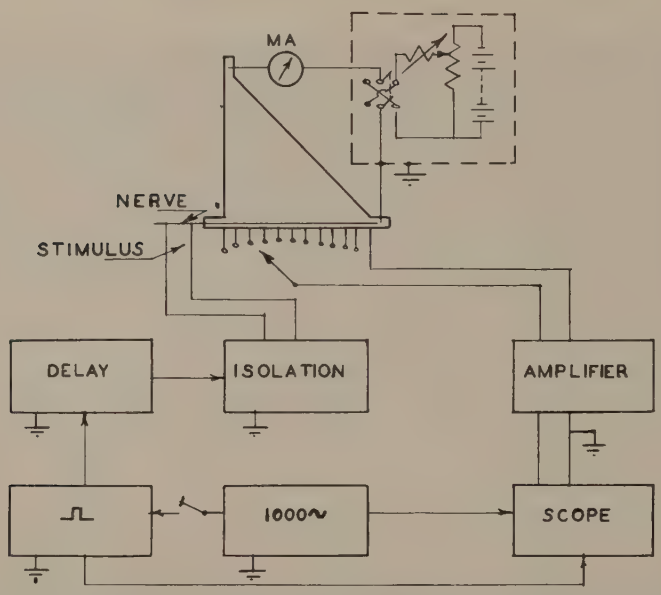

Fig. 3 Block diagram of electrical equipment for polarizing and stimulating nerve and for observing action potential at various points in the polarized region.

the shock artifact was detectable only with difficulty. Fifty microsecond pulses were used throughout.

Action potentials were observed on a Dumont no. 247 oscilloscope after amplification by one unit of a modified Offner electroencephalograph. A 1000 cycle tuning fork oscillator provided millisecond gaps in the oscilloscope trace and locked the stimulator to a submultiple (about 5 per second).

Since the amplifier was capacity coupled, sudden application of the polarizing voltage resulted in amplifier block and

no observations were possible during the transient establishment of the applied membrane current.

Polarizing currents were supplied from a 45 volt dry battery as indicated in figure 3.

3. Application of test solutions

In the determinations of the effect of modification of the ionic environment of the nerve on the curve of conduction

TABLE 1

Composition of solutions used. All values are given in millimols per liter

SOLUTION	NaCl	KCl	$CaCl_2$	NaH_2PO_4	Na_2HPO_4
Normal Ringer	111	1.6	1.8	1.33	5.35
Isotonic KCl	..	111	..	1.33	5.35
½ Isotonic KCl	55	55	1.8	1.33	5.35
10 × Normal $CaCl_2$	102	1.6	18	..	..
½ Isosmotic $CaCl_2$	55	1.6	37	..	..
5 × Isosmotic $CaCl_2$	55	1.6	370	..	..

velocity versus polarizing current, it was necessary to maintain the stimulated portion of the nerve in a reasonably normal solution. If this were not done, the effect of the applied current on conduction would not be clearly distinguishable from the effect on the excitability as such.

Therefore, in all such experiments, the nerve was exposed to the test solution in the following way:

The sciatic nerve in the region of the sciatic plexus was tied off and suspended over the test solution so that only that portion of the nerve which would later be in the polarized region was exposed. The whole assembly was then placed in

a moist atmosphere. It was found that the portion of nerve not in the test solution was not altered by many hours of this treatment.

The compositions of all of the solutions used are shown in table 1.

4. Typical experiment

Two sciatic-peroneal nerves, about 8 cm long, of the frog (Rana pipiens) were dissected, carefully cleaned of excess connective tissue and allowed to rest in normal Ringer solution for one hour. The nerve was then placed in the chamber, crushed at a point about ½ cm from the "indifferent" electrode, the chamber tightly sealed and moist air circulated. Most experiments were conducted at a temperature of 26°C., the maximum variation being from 24°C. to 29°C. The voltage distribution through the chamber was checked and if not correct the filter paper electrode was discarded. A new piece of filter paper was cut for each experiment.

The time scale of the oscilloscope was set at one millisecond per inch and the peak of the action potential read to 1/10 millisecond.

In an effort to minimize the uncertainties due to the action potential being composed of the responses of many fibers, the stimulus was set to stimulate only the most excitable alpha fibers, and maintained subthreshold for the beta group. Since the stimulated locus was outside the polarized region, the effect of the applied current on the effectiveness of the stimuli was minimal. All of the data collected pertain only to the alpha fibers.

The quantity which was measured was the time from the stimulus artifact to the peak of the action potential. With the nerve in air or oil it would be more appropriate to measure the time of the first inflection of the action potential since this would mark the boundary between the active and the inactive region. In the present investigation the nerve was lying on a piece of moist filter paper which made the recording

slightly triphasic and the inflection points lose their special significance.

The polarizing current at each experimental value was left on for about a minute, during which time the peak time of the action potential was recorded at each electrode through the chamber. These values could be repeated in the reverse order, showing that the current flowing for this time did not have a cumulative effect.

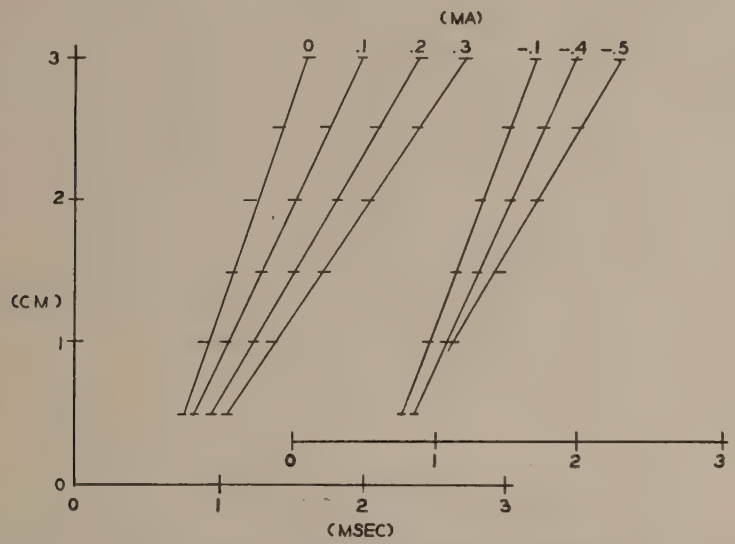

Fig. 4 Method of plotting raw data. Electrode distance versus time of action potential peak. Parameter: total polarizing current.

The data were then plotted with time of action potential versus distance. The velocity through about 3 cm of the polarizing region was constant enough that a straight line could be accurately drawn through the points and the slope (velocity) determined graphically.

RESULTS

1. Normal nerve

A typical plot of distance versus time of action potential peak for various amounts of polarizing current is shown in figure 4. Zero distance refers to the point at which the action

potential peak entered the region of polarization. The stimulating cathode was at minus one centimeter.

The slope of any line in figure 4 is the velocity of the action potential peak of the most excitable alpha fibers for the indicated amounts of applied current. Rheobase was about minus one-tenth milliampere. Positive polarization means the electrode farthest from the nerve was positive, which resulted

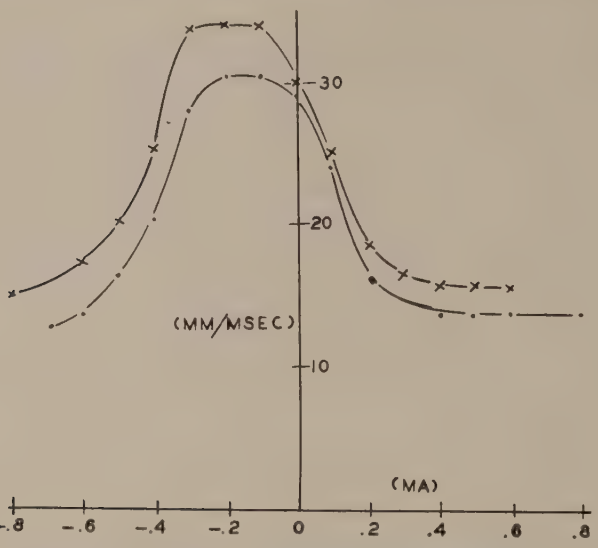

Fig. 5 Typical result. Velocity versus total polarizing current. Two nerves from the same frog. Variation is among the greatest found.

in current flowing inward through the membrane in the plateau region.

The velocities measured from the curves of figure 4 are plotted against the polarizing current in figure 5. The units of velocity are mm/msec.

Figure 5 also shows the results of similar measurements on the other nerve from the same frog. The variation shown is among the greatest found and was usually much less. The total variation with nerves from different frogs can be seen in the results plotted in figure 6. Here is shown the average

curve for measurements taken from 20 nerves and the extreme variation. No experiments were discarded.

The number of values used to average each point in figure 6 was not constant since all nerves did not block at the same value of total polarizing current. More than 50% of the nerves were blocked at the values indicated by the arrows (0.8 ma anodal; 0.7 ma cathodal).

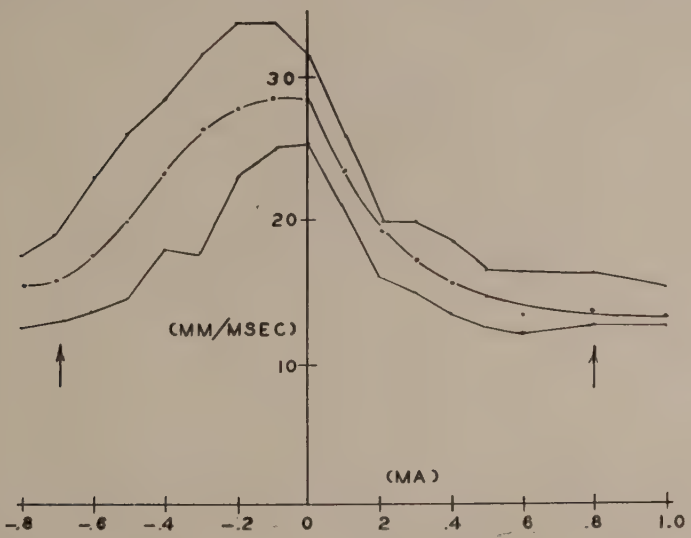

Fig. 6 Average and maximum variation of conduction velocity versus total polarizing current for 20 fresh sciatic-peroneal nerves. Arrows indicate value of polarizing current at which at least half (10) of the nerves were blocked.

It is readily apparent from figure 6 that these results are in agreement with the classical findings of Rutherford and von Bezold, viz., any value of anodal polarization results in a slowing of conduction, while small cathodal currents produce no change, or a slight increase and large cathodal currents produce a slowing.

Block usually occurred at a slightly larger velocity on the cathodal side, but for both anodal and cathodal polarization block occurred at about 50% of the initial, unpolarized velocity.

Of the 20 nerves, 9 had a maximum velocity at zero polarization. Of the remainder, all occurred between zero and minus 0.2 ma. The median of the maxima was at minus 0.5 ma, the average at minus 0.11 ma.

2. Effect of KCl

Nerves soaked in the manner described above in isomolar KCl (0.11 M) for 15 minutes developed total block. The

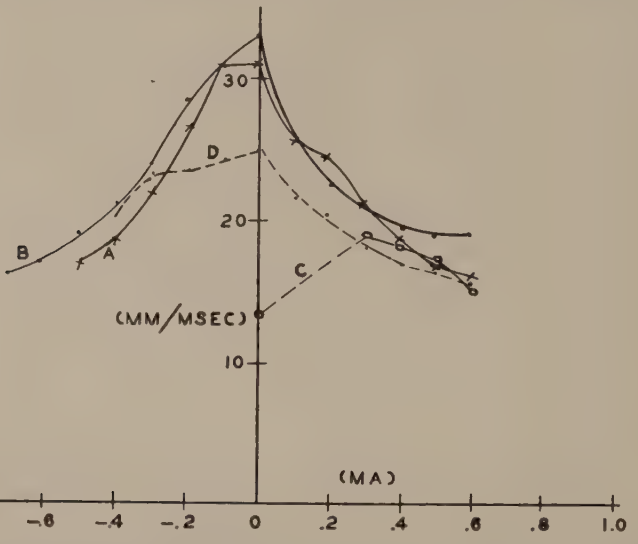

Fig. 7 Chamber control and effect of isotonic (0.11 M) KCl. Conduction velocity versus polarizing current. A, fresh nerve; B, after two hours in chamber; C, after 15 minutes of 0.11 M KCl. Dashed portion of this curve indicates recovery after 10 minutes in chamber. D, recovery after one and one-half hours in chamber (without rinsing).

curve of velocity versus polarization was measured before and after this treatment. It was found that the velocity during anodal relief of the block was not equal to the normal unpolarized value for any amount of applied current, but was equal to about the value that normal nerve had which was subjected to the same degree of polarization.

The results of a typical experiment of this kind are shown in figure 7. In this experiment a nerve was mounted in the

chamber and the usual measurements made. After two hours the measurements were repeated. Following this the nerve was exposed to isomolar KCl for 15 minutes, returned to the chamber and again measured, left in place and remeasured after one and one-half hours.

The first two hours in the chamber did not change the properties of the nerve appreciably. Soaking in KCl caused characteristic changes: total block, relieved by about 0.3 ma applied anodal current; spontaneous recovery in about 10

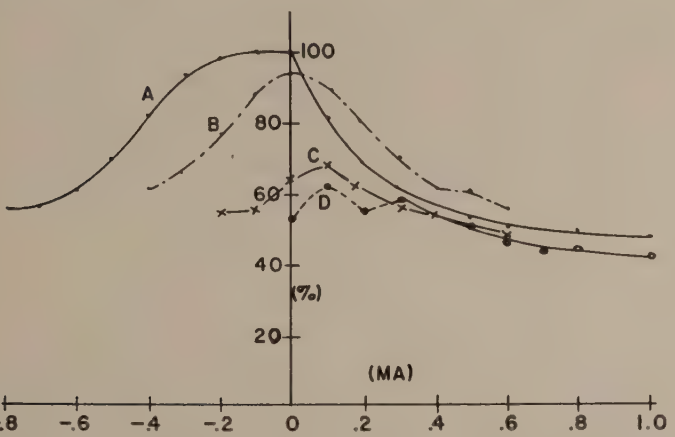

Fig. 8 Effect of isotonic (0.11 M) KCl. Velocity of conduction in per cent of fresh unpolarized velocity versus total polarizing current. A, average normal; B, 5 minutes' exposure; C, 10 minutes' exposure; D, 15 minutes' exposure. Dashed portion of this curve indicates recovery after 10 minutes in chamber.

minutes, at which time the velocity was about one-half normal; slow progressive recovery toward the original state. Removal of the nerve and washing in Ringer solution for 15 minutes restored its properties to normal.

The most surprising aspect of these results is that the velocity for applied currents greater than 0.3 ma was not significantly different from normal (at that level of polarization). This result was true for all such experiments. Occasionally a KCl treated nerve would differ in this region from the normal controls in that much larger anodal currents

were necessary for block. Block occurred, however, at about the same velocity as normal.

Figure 8 shows the results of measurements of velocity against applied current for 5 nerves soaked for 15 minutes in isomolar KCl, three soaked for 5 minutes and one for 10 minutes. These data have been normalized to per cent of normal, unpolarized velocity before averaging. For comparison, the data on the 20 normal nerves has been averaged in the same way.

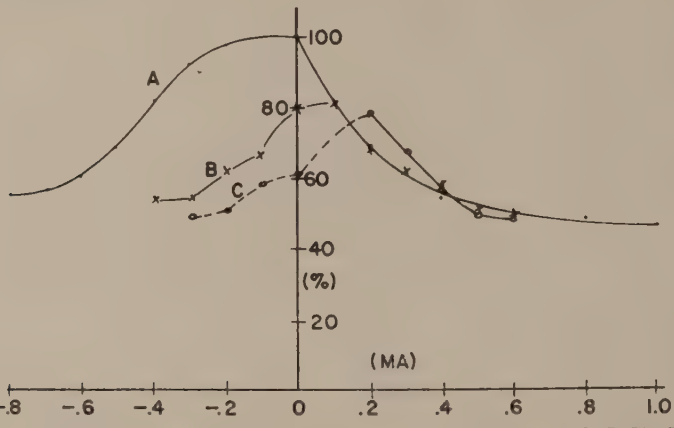

Fig. 9 Effect of one-half isotonic (0.055 M) KCl at expense of NaCl. Velocity of conduction in per cent of fresh unpolarized velocity versus total polarizing current. A, average normal; B, 10 minutes' exposure; C, 15 minutes' exposure. Dashed portion of this curve indicates recovery after 10 minutes in chamber.

Figure 9 shows the same type of results for two nerves soaked in one-half isomolar KCl for 10 minutes, and for two soaked for 15 minutes. It can be seen that the isomolar KCl for 5 minutes had about the same effect as one-half isomolar KCl for 10 minutes. In no case did the velocity fall much below 50% of the normal unpolarized velocity and in no case did the anodal relief restore the velocity to this value.

3. Effect of $CaCl_2$

The compositions of the solutions used are shown in detail in table 1. They will be referred to as normal, 10 × normal. one-half isosmotic and 5 × isosmotic.

At certain stages of block due to $CaCl_2$ it has been reported that the block is reinforced by anodal, relieved by cathodal polarization (Woronzow, '24; Lorente de Nó, '47a, b). Complete block due to calcium is not achieved until drastic histological changes have occurred in the myelin (Lorente de Nó, '47a), and did not occur in any of the experiments reported here, even after soaking for 5 hours in one-half isosmotic $CaCl_2$.

In these experiments the Ringer solution was not buffered. Figure 10 shows the results of soaking nerves in unbuffered

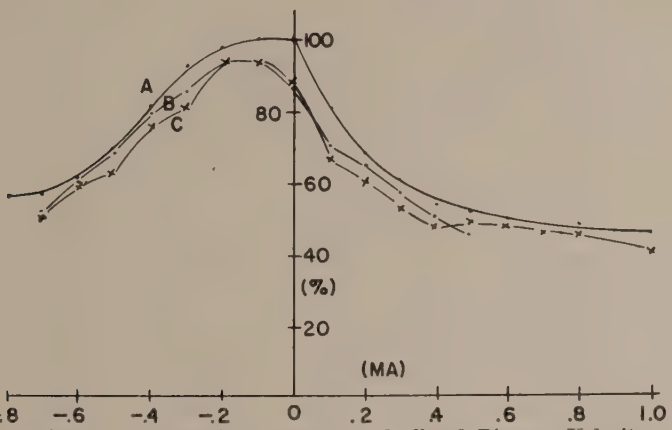

Fig. 10 Effect of prolonged exposure to unbuffered Ringer. Velocity of conduction in per cent of fresh unpolarized velocity versus total polarizing current. A, average normal; B, 3½ hours' exposure to unbuffered Ringer; C, same for 5¼ hours.

Ringer for 3½ and 5¼ hours. This treatment resulted in a slight depression of the velocity at all values of applied current. The effects of high calcium are thus probably not affected by the lack of buffer directly.

Figure 11 shows the results of soaking nerves in one-half isosmotic $CaCl_2$ solution for various times, and, in addition, one experiment with hypertonic (5 × normal) $CaCl_2$. Figure 12 shows the effect of 10 × normal calcium for 15 and 30 minutes.

The initial effect of high calcium was principally a large increase in the value of cathodal polarization necessary for

block (1.5 ma as compared to 0.7 ma), with perhaps a slight shift of the maximum in the cathodal direction. Longer times of soaking or higher values of calcium merely resulted in a lowering of the whole curve with a concomitant decrease in the velocity at the time of block. It is not difficult to see how

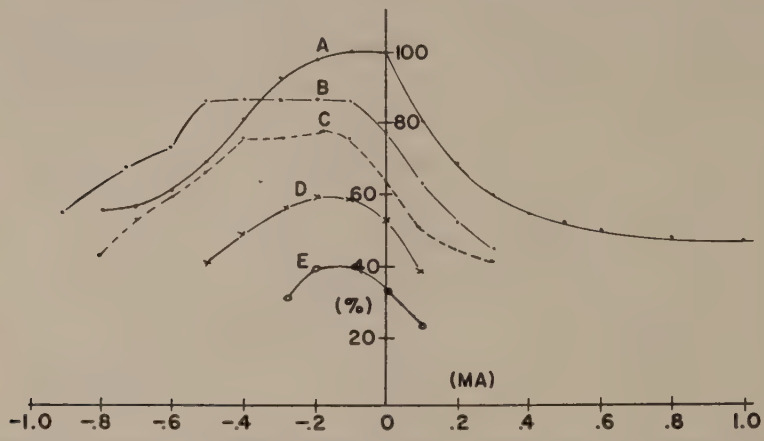

Fig. 11 Effect of one-half isosmotic $CaCl_2$ replacing NaCl. Velocity of conduction in per cent of fresh unpolarized velocity versus total polarizing current. A, average normal; B, one-half hour's exposure; D, 3½ hours' exposure; E, 5¼ hours' exposure; C, 15 minutes' exposure to 5 × isosmotic $CaCl_2$.

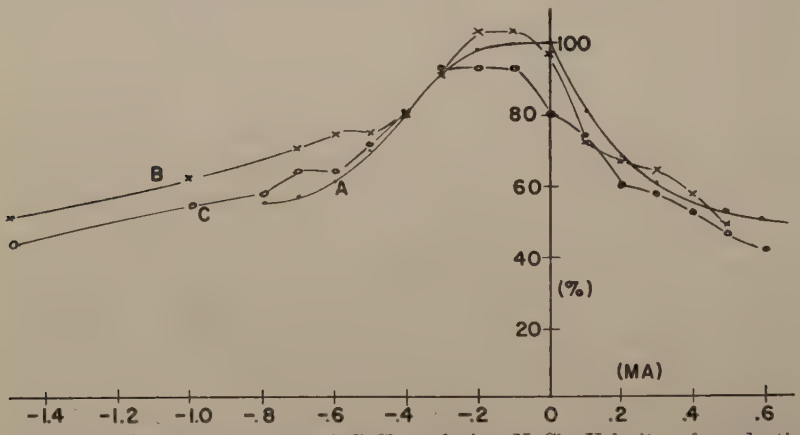

Fig. 12 Effect of 10 × normal $CaCl_2$ replacing NaCl. Velocity of conduction in per cent of fresh unpolarized velocity. A, average normal; B, 15 minutes' exposure; C, one-half hour's exposure.

this behavior might be interpreted as an anodal augmentation of the $CaCl_2$ effect and a cathodal diminution, but this conclusion is clearly untenable.

DISCUSSION

Method

The applied membrane current distribution was inferred from the external applied current and the structure of the nerve trunk. It has been shown (Taylor, '52) that the method of applying current employed would produce a large region of spatially constant membrane current if the nerve were a simple core conductor model. The magnitude of this current in the "plateau" region would be directly proportional to the total current applied to the filter paper electrode (fig. 3) and independent of the resistance of the membrane.

The frog sciatic nerve is not a simple core conductor model! The presence of the resistive epineural sheath has been shown not to interfere in any way, nor does the nodal structure (Taylor, '52). The possible "screening" effect of the peripheral fibers on the central ones should be mentioned. They can be considered as part of the epineural sheath for the more central fibers and, provided that the chamber is long enough and the resistivity of the peripheral fibers as a group does not change too suddenly with distance, they will have no effect on the membrane current distribution of the central fibers. It is emphasized that this is true only for this method of applying current, not for the more usual bipolar method.

It was assumed in the analysis of the saltatory model that the resistance of the myelin sheath was infinite. While this is not strictly true (Huxley and Stämpfli, '49), only the applied radial current through the nodes has any physiological significance (Tasaki, '39), and for the method being discussed the applied nodal currents would reach a plateau even if the myelin had a low resistance. The magnitude of the applied nodal current would not, however, be independent of the nodal membrane resistance.

The effect of the rectifying properties of the nerve membrane has been discussed in the paper referred to above (Taylor, '52) and would be only an indirect one of altering the length of the plateau region.

The uniformity of the velocity of conduction through the mid portion of the chamber strongly suggests that the plateau was occurring in all cases and that the longitudinal currents in the cores of the fibers had no effect on the excitable properties of the membrane, since over the region concerned the internal longitudinal currents increased several fold.

Conduction through a polarized region

When a nerve impulse enters a region of polarization its effectiveness as a means of self-propagation could be altered in three ways: (1) change in the active response; (2) change in the excitable properties of the inactive region; (3) distortion of the *applied* membrane current by the membrane resistance decrease in the active region.

Calculation shows that the third effect could be an important one, resulting in an increase in the effective (and measured) height of the action potential for anodal polarization and a decrease for cathodal, over and above any altered response of the membrane. Indeed, on the basis of present available evidence this effect *alone* could account for the linear dependence of the action potential height on degree of polarization, including the different slopes of this dependence for the anodal and cathodal cases (Schoepfle and Erlanger, '49). In turn, cathodal slowing is well accounted for by the decreased action potential height, and the point of cathodal block is predictable on the assumption of a safety factor of 5 (Tasaki et al., '41).

In an anodal region, alteration of the applied current by the decreased membrane resistance of the active region would be in the direction to increase velocity. Furthermore, under the anode the resting membrane resistance increases and chronaxie decreases (Blair, '36; Bouman, '37), both tending

to increase velocity. Thus the only factor which is in the direction to decrease velocity anodally is the increase in rheobase (Blair, '36; Chweitzer, '35a) to ultimate block of the excitable mechanism.

Effect of KCl. Excess potassium decreases the membrane potential of nerves (Höber and Strohe, see Lorente de Nó, '47a) and increases the conductance of the nerve membrane of the crab (Hodgkin, '47). Both of these effects would be expected to result in a slowing of conduction and ultimate block. Isotonic potassium might be expected to produce block because of the lack of sodium in the medium (Hodgkin and Katz, '49), but in the intact nerve trunk enough sodium would probably be pumped out of the nerve fibers to maintain the concentration in the immediate vicinity sufficiently high for conduction to occur.

It was seen above that during anodal relief of potassium block the velocity did not return to normal. The action potential height was restored to normal. According to Chweitzer ('35b) the rheobase polarization curve is simply translated by excess potassium, with little change in shape. Bouman ('37) has reported little change in chronaxie with potassium increase. It would thus appear that the potassium treated, anodally polarized nerve must still have a low membrane resistance. A decrease in membrane resistance by a factor of three would, calculated on the basis of Rashevsky's theory of conduction ('48), result in a 40% decrease in the velocity of conduction.

Effect of $CaCl_2$. Calcium ion increase does not block frog nerve until it has acted for a very long time. Even in single isolated fibers where the effects of potassium occur in seconds, 10 times normal calcium merely lowered the height of the action potential in 20–30 minutes (Hertz, '47). Block apparently does not occur until the myelin sheaths are irreversibly damaged (Lorente de Nó, '47a, p. 124).

The general picture which arises out of consideration of the effects of high calcium is that the short time effects (less than one hour in intact nerve) are a result of action on the

nerve membrane at the nodes, while the long time effects are due to progressive destruction and increased conductance of the myelin sheath. This would tend to decrease the velocity of conduction and to compress the scale of applied current. This latter would be an artifact, since the nodal membrane current applied would not be as great for the same amount of total applied current as for normal nerve.

These results are consistent with what little has been reported concerning the effect of calcium excess on the various measurable parameters of nerve stimulation. The whole curve of rheobase versus polarization is shifted in the cathodal direction with only a small change in shape (Chweitzer, '35b), the spike height is increased (Erlanger and Blair, '34; Graham and Blair, '47) and chronaxie falls considerably (Blumenfeldt, '25). All of these factors would tend to increase the velocity of conduction except the increase in the rheobase, and the behavior of the rheobase with polarization would explain the shift of the velocity maximum in the cathodal direction.

In this case, as for excess KCl, the difference between the treated and normal velocity-polarization curves would seem to be mainly related to resistance changes; of the nodal membrane for KCl and of the myelin for $CaCl_2$.

SUMMARY

The effects of polarizing currents on the conduction velocity of frog nerve and the modification of these effects by KCl and $CaCl_2$ for short times following the initial transient were studied. A method of externally applying membrane current uniformly over a 3 cm length of nerve was employed.

In this region, the velocity of conduction was uniform for all of the conditions of applied current studied in spite of a very non-uniform distribution of applied longitudinal current inside the fibers. It is concluded that internal longitudinal currents have no effect on the excitable properties of the membrane.

Conduction velocity of normal nerve had its maximum at weak cathodal currents, falling off on either side until block occurred at about 50% of the unpolarized value.

Anodal relief of partial or complete KCl block did not restore the velocity to the normal, unpolarized value, but to about that value which normal nerve had when subjected to the same polarization.

Increased calcium first increased the amount of cathodal polarization necessary for block by 100%. Larger amounts or longer times depressed the velocity and shifted the maximum in the cathodal direction. The velocity near block was about 20–30% of the normal, unpolarized value, for very high calcium.

It is concluded that the lowering of the height of the action potential is the principal cause of cathodal slowing and block, while anodal slowing and block result from an increased rheobase.

It is suggested that while anodal relief of KCl block can be attributed to the artificial increase in the membrane potential, the lowered membrane resistance is not restored.

The effects of high calcium are attributed to some small effect on the membrane excitable mechanism with the bulk of the action being due to a decrease in the resistance of the myelin sheath.

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THE EFFECT OF HELIUM ON THE GAS EXCHANGE OF MICE AS MODIFIED BY BODY SIZE AND THYROID ACTIVITY[1]

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It has recently been shown (Cook, '50; Cook, South and Young, '51) that helium accelerates the oxygen consumption of several animals and tissues, including insects, reptiles and mice. The effect is particularly clear in the laboratory mouse in which the gas exchange of not only the whole animal is increased but also that of diaphragm, liver, ventricle and sarcoma.

Further investigations have been undertaken in order to examine the mechanism whereby helium exerts this effect, attention being paid to both whole animal and tissue metabolism. This report is concerned with the study of whole animals. The animal employed has been the mouse and the approach has been to correlate the extent of helium acceleration with the corresponding level of metabolism in air. Variation in the standard metabolic rate has been achieved in two ways. The first is to measure the effect of helium on the oxygen consumption of two groups of mice which differ in average body size and in the smaller of which the normal gas exchange per unit weight exceeds, as would be expected, that in the larger. The second is to interfere with the endocrine control of metabolism through the thyroid and induce low or high levels of respiration by removing the thyroid or administering thyroxin respectively.

[1] This investigation was carried out with the assistance of Grant No. RG2988(R) from the United States Public Health Service.

The oxygen consumption of the mice was measured manometrically as previously described (Cook, South and Young, '51). Six mice were placed, one each in 6 small wire cages in order to restrict their activity. Each cage was then put in a large jar of approximately 15 liters capacity which contained soda lime and a concentrated solution of KOH as absorbents for carbon dioxide and excess water vapor. The jar was then covered tightly with a plate glass lid and leaks were prevented by a grease seal reinforced by a one kilo lead weight laid on the lid. The gas space in the jar was then connected to an ordinary manometer tube by a glass tube fused into the glass lid and a short, heavy rubber coupling. The pressure was read directly from the manometer. Twenty minutes were allowed for equilibration before readings were taken and thereafter the pressure was recorded every 15 minutes. Periodically oxygen in the chamber was replaced by means of a rubber balloon, filled with oxygen, which was attached through a separate outlet in the plate glass lid. The original atmosphere in the chamber was either air or a mixture of 20% oxygen and 80% helium. In this manner readings could be secured for an indefinitely long period of time without opening or otherwise disturbing the chamber. Usually the measurements were continued for approximately 6 hours. A 7th jar provided a thermo-barometer. The temperature of the room had to be maintained relatively constant by means of a fan heater and a thermostat since the respiration jars cannot be immersed in a water bath. In this way the ambient temperature was kept at 24°C. $\pm$ 1°. The minor fluctuations in temperature were compensated by the thermo-barometer.

Males of the Swiss strain of mouse were used for all experiments, and the animals were maintained on a standard diet developed by the Institute of Experimental Biology, Berkeley, for this purpose, and known locally as the "green diet." The effect of body size was studied by selecting two groups of normal mice, one averaging 25 gm and the other 30 gm, with very little individual deviation from the mean. Altered hormonal conditions were brought about on the one

hand by radiothyroidectomy and on the other by administration of desiccated thyroid. Two groups of animals were used. One group was fed a diet containing 0.2% desiccated hog thyroid and the other group was given I^{131} intraperitoneally, each mouse of the group receiving two successive doses of 175 microcuries each. The oxygen consumption was measured in the thyroidectomized group 5 weeks after the last injection. That of the group fed desiccated thyroid was determined between the third and the 13th day of exposure to the diet.

The data are to be found in tables 1, 2 and 3. The results may be described as follows:

1. The effect of body size

Four sets of normal mice were compared (designated I–IV in table 1), two averaging 25 gm in weight, two averaging 30 gm. One set each of the two weight groups was exposed to air and the other to a mixture of 20% oxygen in helium. Although the 30 gm mice had an inherently lower metabolic rate (ml oxygen consumed per gram per hour) than did those

TABLE 1

Relation between body size and helium effect. All animals were Swiss strain, male mice. Each determination represents oxygen consumption during one mouse-hour

SET NO.	AVERAGE WEIGHT IN GRAMS	NUMBER OF DETERMINATIONS	GAS MIXTURE	MEAN OXYGEN CONSUMPTION IN ML PER GM MOUSE PER HOUR	S. E. OF MEAN
I	25	54	air	2.69	0.0735
II	30	78	air	2.44	0.0530
III	25	78	20% O_2 in he.	3.06	0.0744
IV	30	78	20% O_2 in he.	3.19	0.0636

Relationships between pairs

PAIR	CRITICAL RATIO OF THE MEANS
I and II	2.76
III and IV	1.33
I and III	3.54
II and IV	9.06

weighing 25 gm, the respiration of both groups was raised to approximately the same level upon exposure to helium since there is no significant statistical difference between the values 3.06 and 3.19. On the other hand when we compare, *within each weight group,* the gas exchange of the animals exposed to air with those exposed to helium-oxygen, we find a very significantly higher oxygen consumption in the latter animals. Finally it is clear that the heavier animals, which have a lower standard metabolic rate, show a relatively greater increase in that rate when exposed to helium (from 100% in air to 131% in helium) than the lighter animals (from 100% in air to 114% in helium).

2. *The effect of the thyroid*

(a) Thyroidectomy. The animals in which the thyroid glands had been irradiated by means of I^{131} showed a decrease in standard metabolism of 12.3% with respect to the controls (see table 2). This reduction is not as severe as might be anticipated although the dose was equivalent to one which

TABLE 2

Effect of helium on thyroidectomized mice

SET NO.	TREATMENT OF ANIMALS	NUMBER OF DETERMINATIONS	GAS MIXTURE	MEAN OXYGEN CONSUMPTION IN ML PER GM MOUSE PER HOUR	S. E. OF MEAN
I	Normal	54	air	2.69	0.0735
II	Thyroid out	66	air	2.36	0.0778
III	Normal	78	20% O_2 in he.	3.06	0.0744
IV	Thyroid out	66	20% O_2 in he.	3.27	0.0908

Relationships between pairs

PAIR	CRITICAL RATIO OF THE MEANS
I and II	3.08
III and IV	1.79
I and III	3.54
II and IV	7.60

will completely eliminate thyroid function in rats. It is possible that there is a difference between the two species in susceptibility to radioactive iodine due to the relative size of the thyroid. Alternatively the effect of complete thyroidectomy on metabolic rate may be less in the mouse than in the rat.

Examination of the data indicate that helium raised the oxygen consumption of the thyroidectomized mice to approximately the same level as the normal, or control, group; there

TABLE 3

Effect of helium on mice fed desiccated thyroid

SET NO.	TREATMENT OF ANIMALS	NUMBER OF DETERMINATIONS	GAS MIXTURE	MEAN OXYGEN CONSUMPTION IN ML PER GM MOUSE PER HOUR	S. E. OF MEAN
I	Normal	40	air	2.79	0.1200
II	Fed thyroid	40	air	5.05	0.1350
III	Normal	40	20% O_2 in he.	4.14	0.0991
IV	Fed thyroid	40	20% O_2 in he.	4.82	0.1680

Relationships between pairs

PAIR	CRITICAL RATIO OF THE MEANS
I and II	12.50
III and IV	3.93
I and III	8.67
II and IV	1.07

is little significance in the difference between the mean values 3.27 and 3.06 (critical ratio of the means is 1.79). There were, however, very significant differences between the oxygen consumption of both sets exposed to air with reference to their behavior in helium. Furthermore, the thyroidectomized animals, which have a lower standard metabolism, showed a greater increase in gas exchange upon exposure to helium (from 100% in air to 139% in helium) than the normal animals (from 100% in air to 114% in helium).

(b) Administration of desiccated thyroid. From table 3 it will be noted that the oxygen consumption of the mice fed

thyroid was approximately 81% greater than that of the normal controls. The effect of helium on the controls was to increase the gas exchange from a mean value of 2.79 ml per gram animal per hour to one of 4.14, or in relative terms from a value of 100% in air to a value of 148% in helium. The mean oxygen consumption of the high thyroid animals in air was 5.05 ml per gram per hour. In helium the same animals yielded a corresponding value of 4.82 ml. On a relative basis, if the respiration in air is taken as 100%, that in helium is approximately 95%. The reduction has so little statistical significance (critical ratio of the means is 1.07) that helium must be regarded as having no effect.

A survey of the data presented above makes it reasonably clear that the degree to which helium accelerates the oxygen consumption of mice is a function of the level of the animal's metabolism at the time that helium is substituted for the nitrogen of the air. The relationship is inverse. The lower the standard metabolism of the animal, the greater the increase induced by the presence of helium. The factors which govern the initial metabolic rate appear to be irrelevant, or at least secondary, since the same trend is evident in normal animals of different body size and in animals of the same size but characterized by varying degrees of thyroid activity.

An explanation of the mechanism of this effect must await the outcome of studies with reference to the intrinsic metabolism of the cells and tissues. In the meantime it is worth pointing out that in the case of male Swiss mice there appears to be a ceiling of oxygen consumption in the vicinity of 5.0 ml of oxygen per gram animal per hour. This ceiling can be reached but not exceeded by administering very heavy doses of the thyroid hormone. Under ordinary circumstances the standard metabolism rests at a lower level. The effect of helium is to elevate this level toward the maximum and thus simulate the action of thyroid. Like the thyroid, however, it cannot move the metabolic rate past the upper limit of the physiological reserve. Hence the administration of helium, jointly with desiccated thyroid, cannot produce an accelera-

tion of gas exchange beyond that which has already been accomplished by the hormone.

SUMMARY

Two groups of male mice of the Swiss strain, one averaging 25 gm in weight and the other 30 gm were tested in air and in a mixture of 20% oxygen in helium. Helium accelerated the oxygen consumption of both groups. The degree of acceleration was greater in the heavy group, in which the standard metabolism in air was lower.

Similar experiments were performed with normal mice, mice which had been radiothyroidectomized with I^{131}, and mice fed heavy doses of desiccated thyroid gland. The acceleration of oxygen consumption due to helium was greatest with the slowly metabolizing, thyroidectomized group, intermediate with the normals, and least with the hyperthyroid animals. It is concluded that the effect of helium is inversely proportional to the level of the standard metabolism regardless of the nature of the factors which initially determine that level.

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NOTICE TO CONTRIBUTORS

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